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THE GROWTH OF NEISSERIA MENINGITIDIS IN SIMPLE CHEMICALLY DEFINED MEDIA[1]

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Frantz (1942) described a simple chemically defined liquid medium, containing glutamic acid, cystine, glucose, and inorganic salts for the growth of *Neisseria meningitidis*. He noted that it was beneficial to incubate his cultures in an atmosphere containing supplementary carbon dioxide and stated that the omission of any one of the ingredients from his final medium resulted "in complete failure of growth or its great delay." A similar medium for the meningococcus was reported by Grossowicz (1945). He found that pyruvate and lactate could be substituted for glucose and stated that calcium was an essential ingredient and that cystine was inhibitory.

Certain results obtained during an investigation of the carbohydrate metabolism of the meningococcus indicated that the organism could be cultured from inocula of from 2 to 200 cells per ml in the Frantz medium in the absence of glucose, provided that the cultures were incubated in an atmosphere supplemented with carbon dioxide. Further investigation revealed that under the experimental conditions all of the four strains tested grew well in a Frantz medium containing no carbohydrates or intermediate metabolites of carbohydrates. These results were so different from those cited above that the present experiments were undertaken to seek an explanation of the discrepancy. The basis of the difference appeared to be the occurrence in the parent strains of variants, which were selected by the method of preparing the inoculum. Whereas Frantz used an aqueous suspension of a 6-hour culture on starch agar, we had used a 20-hour subculture in the Frantz medium, inoculated from a culture on blood agar. Our procedure was followed with a view to minimizing the carryover of growth factors from the blood agar. The present experiments indicate that this single subculture resulted in the emergence, from three of the four strains, of variants that were suited to growth in the Frantz medium, regardless of the presence of carbohydrate. On the other hand, when the inocula were prepared from cultures on blood agar, the four strains presented a spectrum of potentialities for growth from small inocula in the Frantz medium: one grew poorly even when glucose was supplied; the second grew well only in the presence of glucose; the third varied from experiment to experiment, sometimes growing well in the absence of glucose, sometimes not; the fourth strain grew readily without carbohydrate. Even after "adaptation," however, growth was always scantier

in the absence of carbohydrate, although it could be initiated regularly with equally small inocula. The strains that had been subjected to repeated passages in the glucose-free medium still retained the ability to ferment glucose and maltose, but not sucrose. Finally, the attribute of growing without carbohydrate seemed to be a stable one in that it was retained after six daily transfers on the medium originally used, namely, blood agar.

MATERIALS AND METHODS

Cultures. All of our strains of *N. meningitidis* were preserved by desiccation from the frozen state and were sealed *in vacuo* in the original all-glass containers. For an experiment, the contents of such a container were taken up in a small volume of Frantz medium and transferred to blood agar plates. No culture was used after more than seven daily subcultures. Unless otherwise specified, all cultures were incubated at 37 C in closed containers in an atmosphere of 97 per cent air and 3 per cent carbon dioxide.

The four strains of meningococcus tested were selected to represent a variety in respect to serological type, virulence, and source. The Chilson strain (type I) was isolated from the cerebrospinal fluid of a patient in the Strong Memorial Hospital in February, 1943. After three daily transfers on blood agar, a 6-hour hormone broth culture was inoculated onto the chorioallantoic membrane of 11-day chick embryos, which died within 24 hours. The infected allantoic fluid was desiccated. Strain 69 (type I) was originally received from the Division of Laboratories and Research of the New York State Department of Health. In the course of prolonged maintenance in our laboratory, it had reverted to the avirulent "stock" form described by Rake (1933), that is, it no longer produced type-specific substance, did not engender type-specific antibodies when injected into experimental animals, tended to autoagglutinate in broth cultures, produced nonmucoid colonies on solid media, and was incapable of infecting mice (when mucin was used as an adjuvant) or chick embryos. A suspension of this organism in normal rabbit serum was desiccated for the present experiments. Strain 520 (type I) was received in the desiccated state from Dr. A. E. O. Menzel in December, 1942. Allantoic fluid of chick embryos infected with this strain was desiccated to provide source material. Throughout the interim, this strain had proved to be a typical virulent type I meningococcus. Strain 1908 (type II-*alpha*) was received from Dr. Sara E. Branham in February, 1944, in the desiccated state. It was transferred several times through chick embryos and was preserved by desiccation of the infected allantoic fluid.

Media. The basal Frantz medium containing no glucose had the following composition: L-glutamic acid hydrochloride, 1.63 g; L-cystine, 0.012 g; $NaH_2PO_4 \cdot H_2O$, 2.5 g, or $Na_2HPO_4 \cdot 12H_2O$, 6.5 g; KCl, 0.09 g; NaCl, 5.5 g; NH_4Cl, 1.25 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; and distilled water to make 1,000 ml. All of the ingredients except cystine and magnesium sulfate were dissolved in 800 ml of water. The cystine was dissolved in a few drops of 3 N HCl and added. The magnesium sulfate was dissolved separately and added last. The pH was adjusted with sodium hydroxide to the range 7.0 to 7.3, and the volume was

brought to 1,000 ml. Glucose was made up separately as a solution containing 25 g per 100 ml. All solutions were sterilized by passage through Selas 02 filters. When needed, the glucose solution was added aseptically at the rate of 2 ml per 100 ml of medium.

Glutamic acid (Eastman) was converted to the hydrochloride, which was re-crystallized three times from 6 N HCl. Cystine (Merck) was purified by dis-solving it in the minimal quantity of 3 N HCl and reprecipitating it by pouring the solution into an excess of water. This process was repeated twice. All other chemicals were Baker's cp analyzed grade.

Experimental procedure. The turbidity of bacterial suspensions was meas-ured by a Klett-Summerson photoelectric colorimeter using filter 42. A scale reading of 40 represented 3×10^8 organisms per ml on the average, as determined by plate counts. The curve was linear up to a scale reading of 60. Plate counts were made by spreading 0.1-ml portions of the appropriate dilutions of culture on blood agar plates. The figures recorded in table 1 represent the counts multi-plied by 45 to give the total number of organisms inoculated per tube.

In the first group of experiments the inoculum was prepared by transferring the culture from blood agar to 25 ml of Frantz medium, with or without glucose as the case might be, in a 125-ml Erlenmeyer flask. These starter cultures were incubated for 24 hours, at the end of which time they usually contained between 10^8 and 10^9 organisms per ml.

In later experiments the inoculum was prepared directly from 20-hour cul-tures on blood agar. The growth was scraped off and homogenized in sufficient Frantz medium (*minus glucose*) to give a suspension containing 2×10^8 or-ganisms per ml.

The tests of the ability of the organisms to grow in various media were based primarily upon the determination of the minimal inoculum that sufficed to initiate growth and secondarily upon measurement of the density of growth. Serial decimal dilutions of the inoculum were prepared in the conventional man-ner by successive transfers of 0.5 ml into 4.5 ml of the test medium, contained in 6- by 3/4-inch test tubes. The presence of growth was judged by the develop-ment of turbidity after 24 and 48 hours' incubation and confirmed in critical tubes by examination of stained smears, by subculture, or by both methods.

RESULTS

Inoculum subcultured in Frantz medium. In table 1 are recorded the data of representative experiments in which the inocula were 20-hour cultures of the or-ganisms in the Frantz medium *containing glucose*. It will be seen that all four strains grew from inocula of 1,000 cells or less, with or without glucose, in the presence of supplemental carbon dioxide. The dependence of the organisms on carbon dioxide is illustrated by the results with strains 69 and 520. In this type of experiment, of course, the possibility could not be ruled out that there was stimulation of growth by glucose or its breakdown products, carried over from the starter culture. This possibility seemed slight in view of the high dilutions used. The tests were repeated, however, using for inocula 24-hour cultures in

Frantz medium *minus glucose*. As measured turbidimetrically, these starter cultures contained only from a third to a tenth as much growth as those prepared in the medium *with glucose*. Nevertheless, the results of the first group of experiments were readily confirmed.

Inoculum taken directly from blood agar: "adaptation." In tables 2 and 3 are presented the data of typical experiments with the Chilson strain and strain 69

TABLE 1

Growth of meningococci in Frantz medium, with and without glucose and with and without carbon dioxide

(Organisms previously subcultured in Frantz medium)

STRAIN	MEDIUM	ATMOSPHERE	DILUTION OF INOCULUM							
			10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9
Chilson	Without glucose	3% CO_2	+	+	+	≉	≉	≉	≉	−
Chilson	With glucose	3% CO_2	+	+	+	≉	≉	−	−	−
			Inoculum 1,395* 45							
69	Without glucose	Air	+	+	−	−	−	−	−	−
69	With glucose	Air	+	−	−	−	−	−	−	−
69	Without glucose	3% CO_2	+	+	+	+	+	+	+	+
69	With glucose	3% CO_2	+	+	+	+	+	+	+	+
			Inoculum 6,300 630							
520	Without glucose	Air	+	−	−	−	−	−	−	−
520	With glucose	Air	+	+	−	−	−	−	−	−
520	Without glucose	3% CO_2	+	+	+	+	+	+	−	−
520	With glucose	3% CO_2	+	+	+	+	+	+	+	+
			Inoculum 45 45							
1908	Without glucose	3% CO_2	+	+	+	+	+	≉	≉	−
1908	With glucose	3% CO_2	+	+	+	+	+	≉	≉	≉
			Inoculum 945 45							

+ = growth present after 24 hours' incubation.

≉ = growth present after 48 hours' incubation but not after 24 hours.

* The number of organisms inoculated per tube was calculated from a plate count made at the start of the experiment.

of meningococci, using inocula taken directly from blood agar. The results show that, under these conditions, the Chilson strain grew in the first subculture from large inocula only, even when glucose was present. In succeeding subcultures, inoculated in each case from the last positive tube of the preceding series after 48 hours' incubation, it was possible to obtain growth from progressively smaller inocula in both media, until from 10 to 100 organisms per tube sufficed. Finally, it was shown that the subculture "adapted" to growth *with glucose* grew from

equally small inocula *without glucose*, and vice versa.   In these and in all similar experiments with the other strains, growth (measured turbidimetrically) in the

TABLE 2

"Adaptation" of the Chilson strain of meningococcus to growth in Frantz medium, with and without glucose

PASSAGE	MEDIUM	DILUTION OF INOCULUM							
		10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8
1*	Without glucose	+	(#)	−	−	−	−	−	−
2	Without glucose	(+)	±	±	−	−	±	−	−
3	Without glucose	+	+	+	#	#	(#)	−	−
4	Without glucose	+	+	+	+	±	+	#	−
4	With glucose	+	+	+	+	+	+	#	#
1*	With glucose	+	(#)	−	−	−	−	−	−
2	With glucose	+	+	+	+	+	−	(#)	−
3	With glucose	+	+	+	+	#	#	#	(#)
4	With glucose	+	+	+	+	+	+	−	#
4	Without glucose	+	+	+	+	+	+	+	−

+ = growth present after 24 hours' incubation.
\# = growth present after 48 hours' incubation but not after 24 hours.
± = turbidity doubtful after 48 hours.   Organisms demonstrated by subculture.
* These series were started with the same suspension in the sugar-free medium of a 20-hour culture of the organism on blood agar, density 2 × 10^8 cells per ml.   Tubes designated by parentheses were the ones used to start the succeeding series.

TABLE 3

"Adaptation" of meningococcus no. 69 to growth in Frantz medium without glucose

PASSAGE	MEDIUM	DILUTION OF INOCULUM							
		10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8
1*	Without glucose	+	(+)	−	−	−	−	−	−
1*	With glucose	+	+	+	#	#	#	−	−
2	Without glucose	(±)	±	±	−	−	−	−	−
2	With glucose	+	+	+	#	#	#	−	−
3	Without glucose	+	+	+	±	+	(+)	−	−
4	Without glucose	+	+	+	#	#	(#)	−	−
5	Without glucose	+	+	+	#	#	#	#	−
5	With glucose	+	+	+	+	#	#	#	#

+ = growth present after 24 hours' incubation.
\# = growth present after 48 hours' incubation but not after 24 hours.
± = turbidity doubtful after 48 hours.   Organisms demonstrated by subculture.
* These series were started with the same suspension in the sugar-free medium of a 20-hour culture of the organism on blood agar, density 2 × 10^8 cells per ml.   Tubes designated by parentheses were the ones used to start the succeeding series.

absence of glucose was only from a fourth to a third as abundant as in the presence of that substrate, even after "adaptation."   The density of the growth ranged from 7 × 10^7 to 4 × 10^8 organisms per ml.

In the case of strain 69, growth in the first subculture occurred readily in the presence of glucose but in its absence almost failed even with the heaviest inocula in the first two passages. Subsequently, the organism became "adapted" to the carbohydrate-free medium.

Similar experiments were conducted with strains 520 and 1908. Both grew in the first subculture from inocula of less than 100 organisms per tube when glucose was present. In four experiments omitting glucose, however, strain 520 grew only from inocula of 10^8 organisms in three cases and from 10^4 in the fourth. Adaptation of this strain to the carbohydrate-free medium was accomplished successfully. In two experiments growth of strain 1908 was initiated in the first passage by inocula of from 10 to 1,000 organisms in the absence of glucose, i.e., essentially as few cells as sufficed in the presence of the sugar.

At the termination of the experiments described above, the cultures grown without glucose were carried successfully through an additional 10 passages in the same medium, 0.05 ml being transferred each time to 5 ml of medium. These cultures were also transferred to phenol red sugar slants (Difco). All four fermented glucose and maltose rapidly, but not sucrose.

Finally, the "adapted" strains of Chilson and 69 were submitted to six daily passages on blood agar and then retested as before for their ability to grow in the carbohydrate-free Frantz medium. (In these tests the inoculum was taken directly from the blood agar plates.) Growth of both strains could still be initiated with inocula of from 10 to 1,000 organisms, indicating that there had been no reversion to the state of the parent strains.

DISCUSSION

The results of the present experiments will be discussed in relation to the problem of definining the nutritional requirements for growth of bacteria. The essence of this problem has been well stated by Wilson and Miles (1946):

> A nutrient may therefore be defined as a substance that is essential either for minimal growth, perhaps under restricted conditions, of an organism trained to be as unexacting as possible, or for optimal growth. The studies of Mueller (1940), for example, on the nutritional requirements of the diphtheria bacillus, have been conducted on the basis of optimal growth, which he defines as the best attainable upon empirically devised media. In the first case, a medium is sought that will promote the growth of at least one of the cells originally inoculated, and the production of a trained culture is most probably due to selection of cells with the greatest synthetic powers. In the second case, not only the reproduction, but the ready and profuse growth, of all the viable cells of the inoculum is aimed at.

Although the possibility of training in the sense of "a direct response to the chemical stimulus of the changed nutrient conditions" (Knight, 1936) cannot be overlooked, our results seem more consistent with the concept that training is "due to the selection of variants which occur under normal conditions in the parent culture" (Dubos, 1945) and which are suited to existence in the altered environment. A series of transfers in media containing progressively smaller

concentrations of "essential" nutrilites was not necessary. Growth in the respective deficient media occurred without delay in the first passage, but only from inocula containing from 10^7 to 10^8 organisms. After not more than two passages, however, growth could be initiated by inocula of from 10 to 1,000 organisms, indicating that most of the cells of the culture were then suited to the deficient media.

If the proposed interpretation were valid, it would be necessary to conclude that the proportion of variants differed in the several strains. Thus, the Chilson strain provided only a small fraction of cells that were able to grow in the Frantz medium even with glucose present; strain 69 comprised a large number that could utilize the complete medium, but only a few capable of growth without glucose; strain 520 appeared to occupy a variable position; and strain 1908 seemed to have developed into a population consisting mostly of individuals not requiring carbohydrate.

Both Frantz (1942) and Grossowicz (1945) have commented upon the apparently simple nutritional requirements of the meningococcus. In view of the copious literature on the subject and of the numerous complex media that have been devised (see, for example, Murray, 1929), it is indeed surprising to discover that this organism can be cultured from minute inocula in very simple media, the composition of which may be said to be exactly defined within the limits of the present standards of chemical purity. The results of the present investigation, however, indicate that these media are not optimal in the sense defined above. This conclusion is supported by unpublished data, accumulated by Dr. Dorothy M. Tuttle in our laboratory, which show that the Chilson strain is not unique in requiring large inocula for initial growth in the complete Frantz medium and that this property is not peculiar to freshly isolated strains. Insofar as our results have general significance, they indicate that meningococci readily produce variants able to grow in simple chemically defined media.

In defining the conditions for the *optimal* growth of a given culture of an organism, it seems necessary to require that it grow in the initial subculture from minute inocula, ideally from a single cell, lest one succeed only in selecting a variant not representative of the majority of the parent population. (It is probable that growth originated from the inoculation of a single cell in at least some of our experiments.) Adoption of such a criterion would still not eliminate the problem of obtaining an inoculum, whether of one cell or of several cells, minimally equipped with growth factors from the original, usually complex, medium. It is well known that bacterial cells may retain effective amounts of such factors despite thorough washing. It was for this reason that our initial experiments employed for inoculum a subculture in the complete Frantz medium. Paradoxically, and apparently owing to the emergence of variants, such organisms, from three of the four strains, were better suited to the deficient medium than those taken directly from blood agar.

In addition to its ability to support growth from the inoculation of a small number of organisms, an optimal medium must yield the largest possible *total*

amount of growth. In this respect, too, the simple media used in the present investigation were suboptimal.[2]

It will be noted from the data in table 1 that our strains were capable of growth in the Frantz medium in the absence of supplemental carbon dioxide, provided that large inocula were used. It seemed of interest to determine whether a variant could be isolated, by the procedure described above, that would be independent of carbon dioxide. Repeated attempts with strain 520 were made without success. Presumably, the growth from the heavy inoculum was made possible by the accumulation of metabolic carbon dioxide.

It may be asked if cultivation of the meningococcus in the simplified media affected such characteristics as type specificity and virulence. Since it is well known that changes in these properties occur spontaneously even on "complete" media (Rake, 1933), it was not thought to be profitable in the present experiments to investigate the question in detail. It has been found, however, that cultures of two type I strains in the Frantz medium provided an abundance of specific polysaccharide (Scherp, 1943).

SUMMARY

Three randomly chosen strains of meningococci, after a brief period of "training," could be cultured from inocula of from 2 to 200 organisms per ml in a liquid medium containing only glutamic acid, cystine, and mineral salts and supplied with carbon dioxide in a concentration greater than that provided by the air. A fourth strain grew under the same conditions without training. The experimental results were consistent with the concept that training resulted from the selection of variants that occurred normally in the parent cultures and were suited to growth in the simplified medium.

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[2] An indication of the nature of this deficiency was obtained in several experiments with the Chilson and 520 strains. Inocula of 50,000 organisms, taken directly from cultures on blood agar, were transferred to 5-ml portions (1) of an "all-purpose" medium, namely, trypticase soy broth (Baltimore Biological Laboratory); (2) of a medium identical in composition to the Frantz medium except that it contained 0.5 per cent of acid-hydrolyzed casein ("vitamin-free" grade, General Biochemicals) instead of 0.13 per cent of glutamic acid; and (3) of Frantz medium with the content of glutamic acid increased to 0.5 per cent. As expected, the Chilson strain failed to develop in the third of these media. It grew rapidly, however, in the other two media. Indeed, both strains grew equally well in these two media and produced from two to six times as dense a growth as had ever been obtained in the Frantz medium under similar conditions. These results indicated that supplementation of the Frantz medium with a mixture of amino acids alone might suffice (a) to meet the nutritional requirements of the Chilson strain and (b) to increase markedly the growth even of a strain (i.e., 520) capable of multiplying readily in the simple medium.

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THE INFLUENCE OF OXYGEN ON NITRATE AND NITRITE REDUCTION

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Since the classical experiments of Gayon and Dupetit (1886), it has been known that oxygen inhibits the reduction of nitrate and the formation of nitrogen by denitrifying bacteria. Several investigators have studied the effect of oxygen on denitrification by more or less qualitative methods, but there has been very little work of a quantitative nature showing the magnitude of the inhibition in relation to the partial pressure of oxygen during growth of the bacteria or during the denitrification process. The present investigation was undertaken to obtain quantitative data on the influence of oxygen on nitrate and nitrite reduction.

LITERATURE

Weissenberg (1897) tested the ability of three denitrifying bacteria to reduce nitrate and nitrite in shallow layers of medium exposed to air and in the complete absence of oxygen. Oxygen was eliminated from the anaerobic cultures by the use of a pyrogallol seal, a hydrogen atmosphere, or glass-stoppered bottles completely filled with the nutrient medium. Weissenberg found that complete denitrification occurred.in the anaerobic cultures, whereas aerobically nitrate was reduced only as far as nitrite. Thus he demonstrated the greater sensitivity of nitrite reduction to inhibition by oxygen. However, Seiser and Walz (1925) observed a considerable nitrogen loss from nitrate-containing cultures of *Pseudomonas putida* exposed to air, though this was less than under anaerobic conditions.

Lloyd and Cranston (1930) measured the gas exchange that occurred when denitrifying cultures were grown in air or in a nitrogen atmosphere in a closed system. They observed a large nitrogen evolution under anaerobic conditions and an almost equally large oxygen uptake in air. They concluded that nitrate was only slightly attacked aerobically, although some nitrogen was lost from the medium even under their most aerobic conditions.

The first quantitative approach to the problem of nitrate reduction was made by Stickland (1931), who determined the influence of oxygen at various partial pressures on the reduction of nitrate to nitrite by cell suspensions of *Escherichia coli*. Under conditions of aeration that should have maintained an equilibrium of oxygen distribution between the liquid and gas phases, he found as little as 0.36 per cent oxygen caused a 21 per cent inhibition of nitrate reduction, 1 per cent oxygen caused approximately 50 per cent inhibition, and 3.76 per cent oxygen caused 93 per cent inhibition. A tenfold increase in nitrate concentration did not modify these results, thus demonstrating that the inhibition was noncompetitive. He found further that carbon monoxide partially relieved oxygen

inhibition of nitrate reduction and concluded that different enzymes are involved in the activation of nitrate and oxygen, since they show different affinities for their substrates and carbon monoxide.

Meiklejohn (1940) investigated the effect of oxygen on denitrification, maintaining that the notion that oxygen interferes with this process is a "neat teleological explanation" never adequately verified. Her experiments were similar to those of Seiser and Walz; using an unidentified strain of *Pseudomonas* she observed that denitrification occurred to almost the same extent in "aerated" and anaerobic cultures. The interpretation of these results is complicated by the fact that the method of aeration was certainly not adequate to keep the culture medium saturated with oxygen at atmospheric pressure. The partial pressure of oxygen in some parts of the "aerated" medium may have been very low.

Van Olden (1940) was the first investigator to apply modern manometric techniques to the study of denitrification. Using *Micrococcus denitrificans* he made the important observation that the ability of washed bacteria to produce nitrogen from nitrate is dependent upon their previous history. Bacteria that had grown anaerobically with nitrate were capable of causing rapid denitrification of nitrate under anaerobic conditions, whereas bacteria grown aerobically either with or without nitrate denitrified very slowly or not at all. Van Olden concluded that "nitrate reductase" is an adaptive enzyme in the sense of Karstrom (1937). It must be pointed out, however, that from his results it is impossible to decide which enzyme or enzymes failed to develop under conditions unsuitable for denitrification. Since nitrite was formed by some of the bacteria grown aerobically, it is quite possible that their inability to denitrify was at least partially due to the absence of a nitrite reductase or some other enzyme mediating a reaction between nitrite and nitrogen.

Lemoigne *et al.* (1946) found that when *Bacillus megatherium* was grown in a medium containing nitrate as the sole nitrogen source, a pure oxygen atmosphere greatly increased the lag period. This did not occur if there was a source of organic nitrogen in the medium or if the atmosphere contained less than 64 per cent oxygen. They concluded that oxygen arrests the mechanism involved in the assimilation of nitrate, a conclusion that seems to harmonize with the findings of Weissenberg (1897) that the reduction of nitrite is especially susceptible to the inhibitory action of oxygen.

Korochkina (1936) reported that high rH values (24 to 25) did not prevent denitrification and concluded that the process probably could not be eliminated by aeration. She observed, however, that in a medium of rH 35 the rate of reduction of nitrate to nitrite was reduced.

Korsakova (1941) recently reported that, when an organic carbon source was supplied in an amount 5 to 10 times in excess of that required to reduce the available nitrate, the reduction of the latter was as complete under "aerobic" as under anaerobic conditions. This result is difficult to interpret in view of the absence of information on the relative rates of nitrate reduction and on the precise conditions of aeration.

With the exception of the work of Seiser and Walz and of Meiklejohn, the evidence summarized above strongly indicates that oxygen has a deleterious effect on the reduction of nitrate and nitrite and that one or more of the enzymes involved in denitrification is adaptive in the sense that it is only formed in bacteria grown anaerobically in the presence of nitrate. The apparent inconsistencies in the literature may be due to differences in the behavior of different bacteria. However, a more likely explanation is variation in methods of maintaining "aerobic" conditions. Unless special precautions are taken to maintain equilibrium between the atmosphere and the culture medium, it cannot be assumed that all parts of the medium are adequately supplied with oxygen.

MATERIALS AND METHODS

The organism used in these studies was isolated from soil after enrichment in a succinate-nitrate medium. It was identified as *Pseudomonas denitrificans* (Breed *et al.*, 1948). The original culture was kept at 3 to 5 C and remained viable for over 3 years. At intervals of 3 to 6 months, the original culture was transferred to fresh slants of peptone-acetate-nitrate (medium A) agar having the following composition in grams per liter: $Na_2C_2H_3O_2 \cdot 3H_2O$, 8.5; KNO_3, 10; Difco peptone, 4; and agar, 20; pH 7.2. After growth at 28 C these slants were maintained at 3 to 5 C and were used as inocula for all cultures needed for the experimental work. Two or three rapid transfers were made in medium A broth before inoculating large liquid cultures used for growing cells for manometric experiments.

Anaerobically grown cells were obtained by inoculating 0.2 to 0.3 ml of a 48-hour culture in medium A broth into 1 liter of the same medium in a deep vessel (volumetric or Erlenmeyer flask). Cells were harvested after vigorous gas evolution was apparent (usually 35 to 40 hours) and were washed in $M/60$ phosphate buffer, pH 7.0. Prior to centrifugation, dissolved gases in the culture medium were removed by transferring the entire culture to a suction flask and applying a vacuum. If this step is omitted, gas bubbles form on sedimented organisms and carry them back to the surface. After being washed, the cells were resuspended in 5 to 8 ml of $M/40$ phosphate buffer, pH 7.0, and centrifuged lightly to remove clumps. The latter operation is important in order to obtain a relatively homogeneous suspension in which all the cells are subjected to a uniform environment.

Cells grown in air were obtained by inoculating the bacteria into 1-liter widemouthed Erlenmeyer flasks containing 300 ml of medium A broth. These flasks were incubated in a shaking machine at 28 C. Cells grown at reduced oxygen tensions were cultivated in 2-liter flasks provided with sintered glass aerators, the desired O_2-N_2 mixture being bubbled through the medium at a rate of approximately 2 liters per minute. Since at this rate the quantity of oxygen passing through the medium was greatly in excess of the requirements of the bacteria and since the gas was very finely-dispersed and the liquid vigorously stirred, it is probable that the partial pressure of oxygen in the medium was very close to that in the gas at all times. A few drops of "neo-fat 17" were added to the

medium to minimize foaming during aeration. Cells grown in the presence of oxygen were always harvested near the end of the logarithmic growth phase, as determined by photoelectric turbidity measurements.

In manometric experiments involving oxygen at less than the atmospheric level, a suitable mixture of oxygen and nitrogen was prepared by the use of flow meters, and the gas was flushed for several minutes through the vessels. The oxygen content of a sample of the gas was usually determined by standard methods of gas analysis. For very low oxygen tensions, however, the pyrogallol methods of Elliot and Henry (1946) were used to estimate the actual oxygen concentration in the experimental vessels. Oxygen-free nitrogen was prepared by passing tank nitrogen over hot copper.

In manometric experiments, the bacteria were suspended in 0.025 M phosphate buffer, pH 7.0. Alkali was present in the center well of each Warburg vessel. Usually 0.2 ml of 0.1 M sodium acetate were used as the oxidizable substrate, and 0.2 ml of 0.04 M sodium nitrate or nitrite were added from the side arm after equilibration. All manometric experiments were done at 37 C.

Nitrite was estimated by the method of Rider and Mellon (1946).

Medium B. Medium B contained the following compounds in grams per 100 ml of distilled water: KNO_3, 5.00; sodium acetate, 4.21; $K_2HPO_4 \cdot 3H_2O$, 3.97; KH_2PO_4, 0.24; $MgSO_4$, 0.057; $CaCl_2$, 0.0087; and sodium glutamate, 0.096. Four volumes per cent of yeast autolyzate were also included.

RESULTS

Preliminary manometric experiments showed that suspensions of bacteria grown under anaerobic conditions cause a rapid denitrification with either nitrate or nitrite. Figure 1 shows that nitrogen was formed from nitrite at an almost constant rate until the nitrite was used up. The slight hump in the curves for both nitrite and nitrate during the first 20 minutes is due to a lag in the absorption of carbon dioxide. With nitrate the rate of nitrogen evolution was at first slower than with nitrite, but it increased gradually until the two rates were nearly equal. In some experiments the difference in rates with the two substrates was not so great, but qualitatively the same effect was always observed. This effect can undoubtedly be ascribed to a competition between nitrate and nitrite as hydrogen acceptors. Since nitrate is always reduced somewhat more rapidly than nitrite, the formation of nitrogen from nitrite is retarded until most or all of the nitrate has been used up.

Inhibition of nitrite reduction by oxygen. The manometric method cannot be used to follow denitrification in the presence of oxygen, because no simple method is available for simultaneously determining nitrogen evolution and oxygen uptake. It was necessary, therefore, to follow denitrification of nitrite by means of nitrite analyses. Since the conversion of nitrite to nitrogen involves several steps, nitrite analyses will only give a true measure of nitrogen evolution when there is no accumulation of intermediate products such as have been detected under special conditions by Korsakova (1927) and Elema *et al.* (1934).

The accumulation of possible intermediates between nitrite and nitrogen was

investigated by an experiment in which the disappearance of nitrite was followed chemically and the formation of nitrogen was determined manometrically. The results, presented in table 1, show that the nitrogen evolved was equivalent
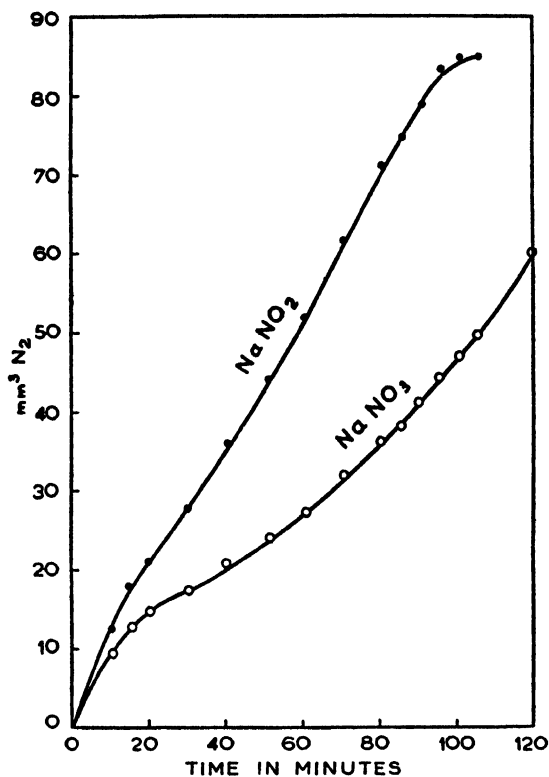
Figure 1. Nitrogen formation from nitrite and nitrate.

TABLE 1

A comparison of nitrite utilization with nitrogen formation

TIME	NO$_2$-N USED*	N$_2$ FORMED
minutes	*μM N*	*μM N*
5	0.4	1.0
40	3.2	2.8
80	5.9	5.1
150	7.4	8.5
215	12.6	12.1
265	15.3	14.7

* The initial quantity of nitrite N was 20.0 μM.

to the quantity of nitrite reduced within the limit of error of the determinations. This means that under the conditions of our experiments there was no significant accumulation of intermediate reduction products.

Preliminary experiments showed that air inhibits denitrification of nitrite by cell suspensions. Before the main experiments to determine the relation between the rate of denitrification and oxygen partial pressure were done, it was important to find out whether the inhibition by oxygen is reversible. If the action of oxygen were irreversible over short periods of time, extreme precautions would have to be taken in handling the bacteria prior to and during the experiment.

To investigate the reversibility of oxygen inhibition, the rates of nitrogen evolution from nitrite by anaerobically grown bacteria were compared manometrically after different periods of exposure to air. In one vessel that served as an anaerobic control, the cells were maintained under a nitrogen atmosphere throughout the experiment. In a second vessel the cells were shaken in air and the rate of denitrification was followed by periodic nitrite analyses. In two other vessels, the cells were shaken in air for different lengths of time; then the air was flushed out with nitrogen and the subsequent anaerobic rate of nitrogen evolution was followed manometrically.

TABLE 2

Reversibility of oxygen inhibition of denitrification

CONDITION	TIME OF RATE MEASUREMENT	RATE OF DENITRIFICATION
	minutes	*mm³ N_2/hr*
Anaerobic	0–160	30.0
Anaerobic following 15-min preaeration	30–160	28.0
Anaerobic following 62-min preaeration	80–160	23.2
Aerobic	0– 80	10.1
	80–135	3.8

The results presented in table 2 show that oxygen inhibition of denitrification is almost completely reversible after a 15-minute exposure to oxygen, but is only partially reversible after 1 hour. It is probable that a much more prolonged exposure to oxygen might cause a permanent inhibition. It should be mentioned that the rate of nitrite disappearance in the vessel continuously shaken with air did not decline continuously, but stayed almost constant for about 60 minutes and then dropped rather suddenly to the lower rate shown in the table. The most important conclusions in relation to further experimental work are that the effect of oxygen is largely reversible over short periods of time and the rate of denitrification responds very rapidly to a change in experimental conditions.

The main experiments were done in the Warburg apparatus, although only with the anaerobic vessels were the manometric data useful for the final calculations. Six vessels were filled with the same cell suspension and reagents, and each vessel was then flushed with a different mixture of oxygen and nitrogen. The vessels were shaken at a rapid rate (150 oscillations per minute) to ensure equilibrium between the gas and liquid phases. The period of incubation was sufficiently short so that the partial pressure of oxygen would not change ap-

preciably during the experiment. The nitrite content of the suspension was determined at zero time and again after a 25- to 50-minute period of incubation. From these results the average rate of nitrite reduction was calculated for each vessel. The results are plotted in the lower curve of figure 2 as a function of the oxygen content of the gas phase. The curve shows that nitrite reduction is inhibited about 45 per cent by 2.5 per cent oxygen. At higher oxygen levels there is a further gradual decrease in rate until in air (20.6 per cent oxygen) the inhibition is 73 per cent. In other experiments the percentage inhibition by air varied from 65 to 75 per cent. Oxygen levels above atmospheric were not used.
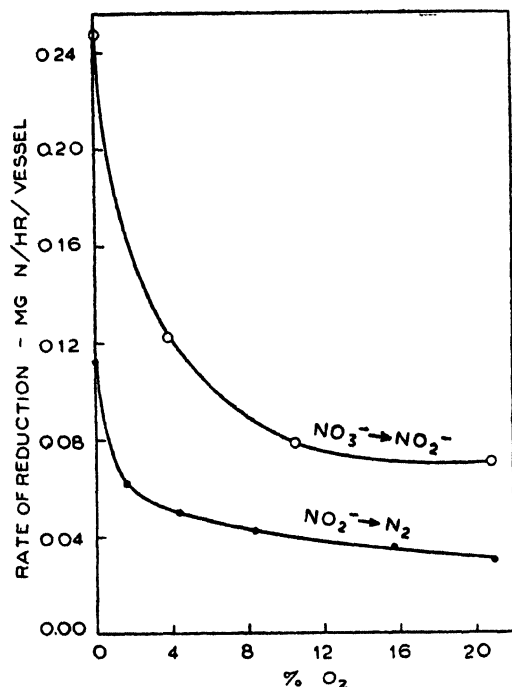
Figure 2. Inhibition of nitrate and nitrite reduction by oxygen at various partial pressures.

Inhibition of the reduction of nitrate to nitrite by oxygen. In a preliminary experiment the effect of air on nitrate reduction to nitrite was determined by measuring the nitrite concentration at intervals in two identical suspensions, one shaken in air, the other in a nitrogen atmosphere. During the sampling of the anaerobic suspension, nitrogen was flushed through the vessel to prevent contamination with oxygen. Parallel analyses were done on suspensions in which nitrate was replaced by nitrite. The results presented graphically in figure 3 show that under anaerobic conditions the reduction of nitrate by cell suspensions of *P. denitrificans* is much faster than the reduction of nitrite, thus causing a temporary large accumulation of nitrite. The rate of nitrite disappearance after the maximum has been passed is almost identical with the rate of nitrogen for-

mation in the anaerobic suspensions initially supplied with nitrite.   In the presence of air, nitrate reduction to nitrite was considerably slower than in nitrogen.   Thus air inhibits the reduction of nitrate as well as nitrite.

To determine the influence of various oxygen tensions on nitrate reduction an experiment similar to the main experiment described for nitrite reduction was performed.   The data gave the rate of nitrite accumulation at various oxygen
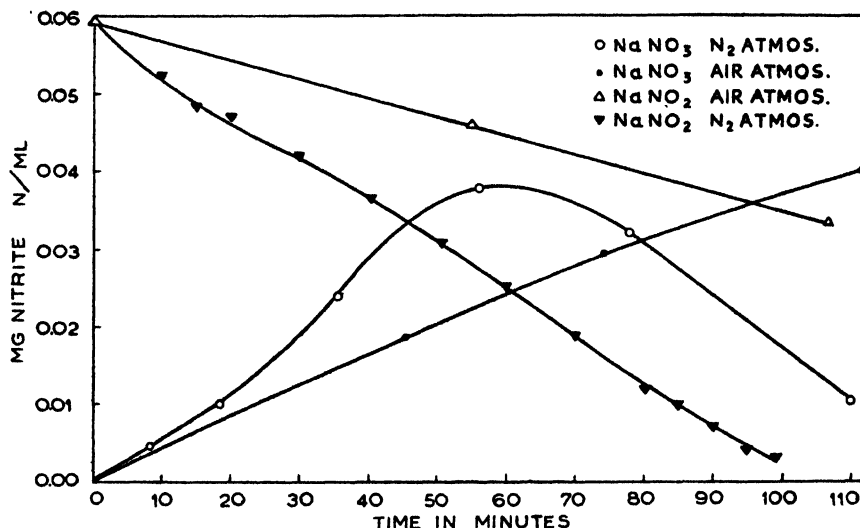
Figure 3. Influence of air on nitrate and nitrite reduction.

TABLE 3

Influence of oxygen on the rate of nitrate reduction to nitrite

1. OXYGEN IN GAS PHASE	2. FRACTION OF ANAEROBIC NITRITE REDUCTION OCCURRING AT THIS O_2 TENSION*	3. RATE OF NITRITE REDUCTION	4. RATE OF NITRITE ACCUMULATION	5. RATE OF NITRATE REDUCTION (3 + 4)
%		*mg N/hr*	*mg N/hr*	*mg N/hr*
0	1.00	0.206	0.043	0.249
3.65	0.46	0.095	0.026	0.121
10.5	0.36	0.062	0.017	0.079
20.8	0.27	0.055	0.016	0.071

* From lower curve of figure 2.

tensions.   Now the rate of nitrate reduction is equal to the rate of nitrite accumulation plus the rate of nitrite reduction.   The latter quantity was calculated from the anaerobic rate of nitrite reduction in this experiment and the percentage inhibition of nitrite reduction at each oxygen tension obtainable from the lower curve in figure 2.   The rate of nitrate reduction estimated in this way is plotted against oxygen concentration in the upper curve of figure 2, and the primary data are given in table 3.   The data results show that nitrate reduction is in-

hibited by oxygen in much the same manner as nitrite reduction. Low oxygen tensions cause a large percentage decrease, but even in a solution fully saturated with air the rate of nitrate reduction is 29 per cent of the anaerobic rate.

Influence of oxygen tension during growth on the formation of nitrate- and nitrite-reducing enzyme systems. Van Olden (1940) observed that denitrifying bacteria cultivated under aerobic conditions were almost or entirely unable to reduce nitrate to nitrogen and concluded that the formation of one or more enzymes essential to denitrification was prevented by air. In view of this observation it was of interest to investigate the ability of bacteria grown under lower oxygen tensions to reduce nitrate and nitrite.
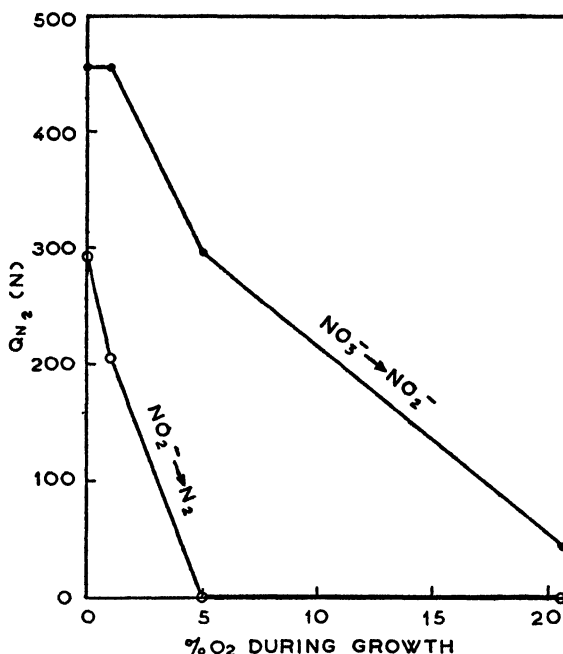
Figure 4. Influence of oxygen concentration during growth on the formation of nitrate- and nitrite-reducing enzymes. The activities of the enzymes were measured under anaerobic conditions.

The bacteria were grown under four conditions of aeration, namely, anaerobically, and aerobically while being vigorously aerated with 1.0, 5.0, or 20.6 per cent oxygen in nitrogen. The cells, harvested at about the same phase of growth, were tested for their ability to reduce nitrate to nitrite and to reduce nitrite to nitrogen under anaerobic conditions. The results presented graphically in figure 4 show that the oxygen tension during growth has a tremendous effect upon the rates of both processes. The formation of nitrite-reducing enzymes is decreased 29 per cent by 1 per cent oxygen and is completely prevented by oxygen at a level of 5 per cent or higher. The lowest oxygen level capable of preventing the formation of nitrite-reducing enzymes may be considerably below 5 per cent; no data are available in the range between 1 and 5 per cent oxygen. The for/

mation of nitrate-reducing enzymes is much less sensitive to oxygen during growth; 1 per cent oxygen causes no detectable inhibition and even saturation of the culture medium with air does not completely prevent the formation of such enzymes.

Stability of denitrifying capacity under various conditions. There is some evidence in the literature (Giltay and Aberson, 1892) that denitrifying bacteria gradually lose their ability to reduce nitrite as a result of being grown under laboratory conditions. It seemed desirable therefore to find out whether our culture would undergo such degeneration and whether the change, if it occurred, was influenced by the availability of oxygen.

To investigate these questions, bacteria that had been grown in medium B for a considerable time under (a) aerobic and (b) anaerobic conditions were tested from time to time for their ability to denitrify. In the aerobic series 10 successive daily transfers were made in small Erlenmeyer flasks incubated on a shaker to ensure adequate aeration. In the anaerobic series 16 successive daily transfers were made using test tubes provided with pyrogallol, potassium carbonate seals.

The ability of the bacteria in each culture of the aerobic series to denitrify was measured by inoculating them into an anaerobically incubated medium and determining the growth turbidimetrically after 48 hours. Since, under anaerobic conditions, denitrification coupled with the oxidation of organic compounds was the only energy-yielding process available to the bacteria, it could be assumed that a decreased ability to denitrify could be reflected by a decreased rate of growth. Although there was some variation in the turbidity of different cultures, there was no consistent trend in the results. The last two cultures in the series grew just as rapidly as the first two. These results indicate that no marked decline in denitrifying ability occurred as a consequence of repeated subculturing under strongly aerobic conditions.

This conclusion was further substantiated by comparing the pH changes that occurred when the bacteria from the final cultures of the aerobic and anaerobic series were grown for 48 hours in medium B under anaerobic conditions. The pH change may be taken as a direct measure of the amount of denitrification. The observed increase, from pH 6.8 to 8.3, was the same in both cultures. Also nitrite was absent from both cultures. These results confirm the conclusion that the denitrifying capacity of the organism is independent of its previous history of exposure to oxygen.

DISCUSSION

The experimental results clearly show that oxygen affects nitrate reduction and denitrification in two ways, by suppressing the formation of the enzyme systems that catalyze these reactions and by directly interfering with the action of the enzyme systems when they are present in the bacteria. The first effect is the more striking in the organism we have studied, but both effects are of great importance in determining the actual rate of these processes.

Exposure of the bacteria to oxygen during growth suppresses the formation

of enzyme systems responsible for nitrite reduction much more than those responsible for the reduction of nitrate to nitrite. As a result, at oxygen tensions of about 5 per cent, nitrate can be reduced only as far as nitrite, which accumulates in the medium. At lower oxygen tensions there is also an abnormally large accumulation of nitrite, but this is accompanied by denitrification. In this range of oxygen tensions, both the accumulation of nitrite and the rate of denitrification are greatly affected by relatively small changes in the oxygen level. In view of these relations it is easy to understand why apparently contradictory results have been obtained in the past by different investigators who used different methods of aerating their cultures.

Under natural conditions such as exist in the soil or in composts the occurrence of denitrification is generally dependent upon the formation of nitrite or nitrate by nitrifying bacteria. Since nitrification requires oxygen whereas denitrification is generally thought of as an anaerobic process, there has been some question as to whether both can occur simultaneously under the same conditions. Our results, taken in conjunction with the data of Meyerhof (1917) on the relation between oxygen partial pressure and the rate of nitrification, indicate that at oxygen tensions below 5 per cent this is possible. In a region of higher oxygen tension, denitrification will not occur together with nitrification unless the bacteria have been able to move from an adjacent region of lower oxygen tension within a short period of time. In this connection it should be emphasized that in a heterogeneous system such as soil the oxygen tension may change greatly over very short distances. Under such circumstances the diffusion of nitrite and nitrate as well as the movement of bacteria will be a factor in determining the rate of denitrification.

SUMMARY

Oxygen has a twofold action on denitrification: it suppresses the formation of nitrate- and nitrite-reducing enzyme systems, and when these systems are present it decreases the rate of the reduction processes. Quantitative data are given illustrating both of these effects of oxygen. It has been shown also that the inhibition of denitrification by air is largely reversible over short periods of time. Ability to denitrify did not decline appreciably as a result of continuous growth under aerobic conditions.

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DEMONSTRATION OF AN INTERFERENCE PHENOMENON ASSOCIATED WITH INFECTIOUS BRONCHITIS VIRUS (IBV) OF CHICKENS

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Infectious bronchitis of chickens is a widespread, highly contagious respiratory disease of high morbidity but negligible mortality and one that causes considerable economic loss due to retardation of growth and reduced egg production (Beach, 1945). The causative agent, a filterable virus, was first isolated in chicken embryos by Beaudette and Hudson (1937). These investigators found that the egg-adapted strain of IBV was lethal for chicken embryos after several passages and that no gross lesions were produced on the chorioallantois although infected embryos were found to be considerably smaller than comparable normal embryos or embryos infected with fowl pox. Delaplane and Stuart (1941) have reported that the virulence of IBV for chicken embryos increased with continued passage in eggs but that the egg-adapted strain ceased to be virulent for chicks or immunogenic for chickens after 89 embryo passages. However, egg-adapted IBV has been used in neutralization tests for the measurement of antibody levels in serum and egg yolk in both experimental and field outbreaks of the disease (Jungherr and Terrell, 1948). During the routine passage of egg-adapted IBV in this laboratory it was observed that embryos inoculated with 0.2 ml of undiluted allantoic fluid from dead embryos frequently did not die until the fourth to the sixth day after inoculation, but embryos inoculated with the same fluid diluted 10^{-1}, 10^{-2}, or 10^{-3} were dead by the second or third day after inoculation. This observation suggested the possibility of the existence of an interference phenomenon somewhat similar to the interference of inactive virus with the propagation of the influenza viruses in the chicken embryo described by Henle and Henle (1943). The interference phenomenon, in which the presence of one virus interferes with the propagation of another, not necessarily related, is now well known, and examples too numerous to cite here have been described within the animal, plant, and bacterial virus groups.

MATERIALS AND METHODS

The egg-adapted strain of IBV used throughout these studies was obtained through the courtesy of Dr. C. H. Cunningham and represents a substrain of the original virus first isolated by Beaudette and Hudson (1937). This strain has been maintained in this laboratory for over 125 passages in chicken embryos by inoculating 0.2 ml of a 1:10 dilution of allantoic fluid harvested from dead or moribund embryos into the allantoic sac of normal embryos. Eggs in the

tenth or eleventh day of embryonic development were inoculated into the al-
lantoic sac in the usual manner (Beveridge and Burnet, 1946) and were candled
daily for 6 days after inoculation. All embryos dead on the first day after in-
oculation were routinely discarded. Inoculated eggs were incubated at 36 C
and all inocula were tested for bacterial sterility by incubating 0.5-ml amounts
for 48 hours in tubes of N.I.H. thioglycolate broth (Difco). Living embryos
(both normal and infected) were killed by chilling the eggs at 4 C for 18 hours
before the allantoic fluid was harvested (to obtain fluid free from blood) or before
further incubation of the eggs after the death of the embryo. Infectivity ti-
trations were conducted in the usual manner by inoculating 0.2-ml amounts
(unless otherwise indicated) of serial 10-fold dilutions of a given preparation into
the allantoic sac of groups of 6 or more chicken embryos. The 50 per cent end
point was calculated according to the method of Reed and Muench (1938).

EXPERIMENTAL RESULTS

Propagation of IBV in the chicken embryo. Before attempting to use the
developing chicken embryo as an experimental animal for the study of IBV, it
was desirable to follow the multiplication of the virus in infected eggs. In study-
ing the distribution of IBV in chicken embryos infected by way of the allantoic
sac, Cunningham and El Dardiry (1948) found that the greatest concentration
of IBV occurred in the chorioallantois, followed in order by the allantoic fluid,
amniotic fluid, and liver. In addition, these investigators observed that (a) the
infectivity titer of allantoic fluid from living embryos was higher than com-
parable fluid from dead embryos, (b) the titer of allantoic fluid from living
embryos increased in direct proportion to the length of the postinoculation period
(36 hours), and (c) the titer of allantoic fluid from dead embryos decreased in
direct proportion to the length of the postinoculation period (48 hours). Data
obtained in this laboratory were in substantial agreement, although it will be
seen in the experiment described below that the LD_{50} of fluid from embryos still
living on the third day after inoculation was lower, rather than higher, than that
of fluid from living embryos on the first day after inoculation. In order to fol-
low the concentrations of IBV in allantoic fluid during the first 3 days after in-
oculation 150 eggs were inoculated into the allantoic sac with 0.2 ml of a 1:10
dilution of the same pool of infected allantoic fluid. Allantoic fluid was har-
vested and pooled from groups of living as well as dead embryos on the first,
second, and third day after inoculation, respectively, and titrated in chicken
embryos in the usual manner (see "Materials and Methods").

It will be seen from the data presented in table 1 that (a) the infectivity titer
was highest ($10^{-7.6}$) in allantoic fluid harvested from living embryos on the first
day after inoculation, and (b) the infective titer of allantoic fluid harvested from
embryos living on the second and third day after inoculation was approximately
10 times higher than that of comparable fluid from dead embryos. Under the
conditions of this experiment it would appear that the concentration of active
IBV in allantoic fluid was highest during the first day after inoculation and was
reduced by further incubation of viable embryos as well as by death of the

embryos themselves. It is of some interest to note in this connection that Cunningham and Stuart (1947) have reported that allantoic fluid harvested from embryos receiving 0.05 ml of undiluted fluid per egg had a slightly higher infectivity titer than fluid from embryos receiving 0.1 and 0.2 ml per egg, whereas Cunningham and El Dardiry (1948) failed to find any advantage in the use of fluid diluted 10^{-3} as compared with undiluted fluid. Data obtained in this laboratory have shown that 4 serial passages of 0.2 ml of undiluted allantoic fluid from embryos dead less than 5 hours resulted in fluid with an infectivity titer of $10^{-6.2}$ as compared with the third serial passage of 0.2 ml of fluid diluted 10^{-3} in which the infectivity titer of the resulting allantoic fluid was $10^{-5.7}$. Thus it would appear that viability of the embryo and time of harvest (see table 1) were more important factors to be considered in obtaining allantoic fluid of high infectivity than the concentration of IBV in the inoculum.

Although practical considerations make inoculation by the allantoic sac route the method of choice, it was of some interest to study the effect of IBV on the

TABLE 1

Propagation of IBV in allantoic sac of chicken embryos

ALLANTOIC FLUID HARVESTED FROM EMBRYOS	NUMBER OF EMBRYOS HARVESTED	LD₅₀ OF ALLANTOIC FLUID
L-1	10	$10^{-7.6}$
L-2	10	$10^{-6.7}$
L-3	7	$10^{-6.8}$
D-2	6	$10^{-5.6}$
D-3	7	$10^{-5.7}$

L-1 = embryos living on first day after inoculation.
D-2 = embryos dead on second day after inoculation.

chicken embryo following inoculation onto the chorioallantois. Accordingly, 0.05 ml of infected allantoic fluid were inoculated onto the chorioallantois of 25 embryos in the tenth day of embryonic development, and the eggs were candled daily for 6 days after inoculation. Three inoculated eggs were sacrificed daily and the chorioallantoic membranes were examined. It was found that (a) all embryos not sacrificed were still alive on the sixth day after inoculation, and (b) flat, grayish, pin-point-sized lesions were found on the chorioallantois on the second day after inoculation and were found to increase in size to a maximum of 0.5 mm by the fourth day after inoculation. The infectivity titer of a pool of 2 chorioallantoic membranes harvested 2 days after inoculation onto the chorioallantois was found to be $10^{-6.5}$. It is evident that inoculation of IBV onto the chorionic side of the chorioallantois was followed by multiplication of the virus and the formation of minute lesions on the membrane but did not result in death of the embryo.

Induction of the phenomenon by further incubation of infected eggs after death of the embryo. During the routine passage of egg-adapted IBV in this laboratory

it was observed that embryos inoculated with 0.2-ml amounts of undiluted allantoic fluid harvested from dead embryos frequently did not die until the fourth to the sixth day after inoculation into the allantoic sac, whereas embryos inoculated with the same fluid diluted 10^{-1} through 10^{-3} were dead by the second or third day after inoculation. However, this phenomenon was not observed in any of many similar titrations made with allantoic fluid harvested from living embryos or from embryos dead less than 2 hours. These observations indicated the possibility that interference might be induced by further incubation of infected eggs at 36 C after death of the embryo. In order to test the validity of this hypothesis the following experiment was conducted. Eight eggs, dead on the third day after inoculation with IBV, were stored for an additional 24 hours at 36 C. The allantoic fluid was then harvested, tested for bacterial sterility, and pooled. As a control, 8 additional eggs, dead for less than 2 hours on the third day after inoculation with the same material, were stored for 24 hours at 4 C before the allantoic fluid was harvested, tested for sterility, and pooled. These two pools of allantoic fluid harvested from eggs stored for 24 hours at 36 C and 4 C, respectively, after death of the embryo were titrated in chicken embryos by the allantoic sac route, and the results are presented in sections A and B of table 2. It is evident from the data presented that storage of infected eggs for 24 hours at 36 C after death of the embryo reduced the LD_{50} of the allantoic fluid approximately 10-fold and markedly decreased the rate of death of embryos inoculated with 1.0 ml of undiluted fluid harvested from such embryos as compared with that of embryos inoculated with the same fluid diluted 10^{-1} through 10^{-3}. It was also found that when allantoic fluid was diluted 10^{-1}, 10^{-2}, or 10^{-3} the time in days required to kill 70 per cent of the inoculated embryos (T_{70}) was identical (3 days). It is of interest to point out in this connection that in each of 4 additional titrations in which a similar interference was observed the LD_{50} of such allantoic fluids was $10^{-4.0}$ or less. That the delay in the rate of death of embryos inoculated with undiluted allantoic fluid harvested from infected eggs stored at 36 C for 24 hours after death of the embryo was correlated with the amount of such fluid inoculated is evident from the data presented in section C of table 2. It will be seen that the T_{70} was progressively decreased from 6 days, in the case of those embryos receiving 2.0 ml of undiluted fluid, to 3 days, in the case of those embryos receiving only 0.25 ml of the same fluid, and that the T_{70} of the embryos inoculated with 0.25 ml of the same fluid diluted 10^{-1} was 2 days.

In the experiments described below attempts were made to reproduce this interference phenomenon by the following methods: first, by storage of infected allantoic fluid *in vitro* for 24 hours at 36 C; second, by storage of infected allantoic fluid *in vitro* in the presence of normal chorioallantoic membranes for 24 hours at 36 C; and third, by dilution of IBV in allantoic fluid harvested from normal embryos killed by chilling in the thirteenth day of development and stored at 36 C for 24 hours before harvest. In the first experiment, half of a pool of allantoic fluid harvested from 20 living embryos on the second day after inoculation was stored at 36 C for 24 hours and the remaining half was stored at 4 C. Fol-

lowing storage at 36 C and 4 C, respectively, both fluids were tested for bacterial sterility and 1.0-ml amounts of each undiluted fluid and the same fluid diluted 1:20 in broth were inoculated into the allantoic sac of groups of 30 embryos. It was found that the T_{70} of all groups was identical (2 days). It would appear, therefore, that interfering material was not produced by incubation of infected allantoic fluid *in vitro* for 24 hours at 36 C.

In the second experiment, allantoic fluid was harvested from 30 living embryos on the second day after inoculation and 10 ml of the fluid were placed in each of 10 test tubes. Chorioallantoic membranes from normal viable embryos in

TABLE 2

Titration of allantoic fluid from eggs stored at 36 C and 4 C after death of the embryos

SEC-TION	INOCULUM					DEAD/ TOTAL	CUMULATIVE PER CENT SURVIVAL OF EMBRYOS INOCULATED						T_{70}*
	No. of eggs	Storage of eggs before harvest	Allantoic fluid				Days after inoculation						
			LD_{50}	Volume inoc.	Dil'd		1	2	3	4	5	6	
				ml									*days*
A	8 D-3†	24 hours at 4 C	$10^{-5.2}$	1.0	Undil.	27/27	100	48	30	15	0	0	3
			$10^{-5.2}$	1.0	10^{-1}	29/29	100	41	14	0	0	0	3
			$10^{-5.2}$	1.0	10^{-2}	29/29	100	52	17	3	0	0	3
			$10^{-5.2}$	1.0	10^{-3}	30/30	100	70	20	7	0	0	3
B	8 D-3	24 hours at 36 C	10^{-4}	1.0	Undil.	19/28	100	96	96	75	61	32	>6
			$10^{-4.0}$	1.0	10^{-1}	25/25	100	44	20	0	0	0	3
			$10^{-4.0}$	1.0	10^{-2}	28/28	100	50	18	0	0	0	3
			$10^{-4.0}$	1.0	10^{-3}	29/30	100	57	13	10	3	3	3
C	40 D-3	24 hours at 36 C	n.t.	2.0	Undil.	15/21	100	95	67	52	43	29	6
			n.t.	1.0	Undil.	22/24	100	92	71	29	21	8	4
			n.t.	0.5	Undil.	25/25	100	72	32	8	4	0	4
			n.t.	0.25	Undil.	24/24	100	54	21	8	4	0	3
			n.t.	0.25	10^{-1}	24/24	100	4	0	0	0	0	2

n.t. = not tested.

* T_{70} = time in days required to kill 70 per cent or more of the inoculated embryos.

† 8 D-3 = 8 embryos dead 3 days after inoculation.

the twelfth day of development were harvested, and one membrane was placed in each of the tubes containing 10 ml of infected allantoic fluid. These tubes were then incubated at 36 C for 24 hours, after which all tubes were tested for bacterial sterility. All sterile fluids were then pooled after discarding the membranes, and 1.0-ml amounts of the undiluted fluid and the same fluid diluted 1:20 were inoculated into groups of 40 embryos by the allantoic sac route. It was found that the T_{70} in both groups of inoculated embryos was identical (3 days). It would appear that interfering material was not produced by the incubation of infected allantoic fluid *in vitro* in the presence of normal chorioallantoic membranes at 36 C for 24 hours.

In the third experiment, allantoic fluid from 8 embryos dead on the third day after inoculation was pooled and diluted 1:40 in the following diluents: (1) tryptose broth, (2) allantoic fluid from normal embryos killed by chilling on the thirteenth day of development, and (3) allantoic fluid from normal embryos killed by chilling on the thirteenth day of development and stored for 24 hours at 36 C before harvest. Following storage at 4 C for 18 hours, 1.0-ml amounts of each of the 3 preparations described above were inoculated into groups of 30 embryos by way of the allantoic sac. It was found that the T_{70} of the 3 groups of inoculated eggs was identical (3 days). It would appear that interfering material was not produced in allantoic fluid of normal embryos by storage of the eggs at 36 C for 24 hours after the embryos had been killed by chilling. Thus it is evident from the experiments described above that no interfering material could be demonstrated (a) in infected allantoic fluid when that fluid was incubated *in vitro* at 36 C for 24 hours alone or in the presence of normal chorioallantoic membranes, or (b) in normal allantoic fluid when normal embryos were incubated at 36 C for 24 hours after the embryos had been killed by chilling.

Effect of heat on infected allantoic fluid. In studying the interference of inactive virus with the propagation of active influenza viruses Henle and Henle (1944) and Ziegler, Lavin, and Horsfall (1944) found that inactivation of infective allantoic fluid by heat as well as by irradiation with ultraviolet light was capable of producing interference with the multiplication of active influenza viruses in the chicken embryo. In view of these findings with the viruses of influenza it was of considerable interest to determine the effect of heat on IBV. Accordingly, infected allantoic fluid was harvested and pooled from living embryos on the second day after inoculation as well as from embryos dead on the third day after inoculation and stored for 24 hours at 36 C before harvest. Aliquot portions of both pools were placed in a water bath at 56 C and removed after 5, 10, 20, and 30 minutes, respectively. Groups of chicken embryos were then inoculated into the allantoic sac with 1.0 ml of these heated fluids together with the respective unheated fluids and with 1.0 ml of the same fluids diluted 1:20. It will be seen from the data presented in table 3 that (a) a temperature of 56 C for as long as 30 minutes did not completely inactivate IBV, (b) the concentration of active virus in fluid from living embryos was reduced to approximately 0.001 per cent (see table 1) when such fluid was heated for 10 minutes at 56 C, and (c) when fluids from dead embryos (containing interfering material) and fluids from living embryos were heated for 10 minutes at 56 C none of 12 embryos inoculated with the former died, whereas 10 of 15 embryos inoculated with the latter died. It would appear from the data presented (table 3) that a temperature of 56 C for 10 minutes was effective in inactivating undiluted allantoic fluid only when such fluid contained interfering material before it was heated.

It has been shown (table 3) that undiluted allantoic fluid from dead embryos stored for 24 hours at 36 C before harvest was not infective for chicken embryos after the fluid was heated for 10 minutes at 56 C. Such heat-treated allantoic fluid from infected embryos will hereafter be referred to for convenience

as inactive virus and comparable heat-treated fluid from similarly treated normal embryos as inactivated normal fluid. In the following experiment IBV was diluted 10^{-3} in inactivated normal fluid and inactive virus, and 1.0-ml amounts of each preparation were inoculated into the allantoic sac of groups of 30 chicken embryos. It is evident from the data presented in table 4 that when IBV was diluted in inactive virus the rate of embryo deaths was strikingly slower (T_{70} = greater than 6 days) than when IBV was similarly diluted in inactivated normal fluid (T_{70} = 3 days). It is also clear from the data presented

TABLE 3

Effect of heat on the infectivity of allantoic fluid from living and dead embryos

ALLANTOIC FLUID HEATED AT 56 C FOR	ALLANTOIC FLUID HARVESTED FROM			
	Living embryos stored 24 hours at 4 C		Dead embryos stored 24 hours at 36 C	
	Undiluted	1/20	Undiluted	1/20
minutes				
0	15/15*	13/14	6/13	14/14
5	14/14	12/15	1/13	2/13
10	10/15	4/15	0/12	2/15
20	8/15	3/15	2/15	1/14
30	7/13	1/13	3/14	1/15

* Number of embryos dead/total number inoculated.

TABLE 4

Inoculation of IBV diluted in inactivated normal fluid or in inactive virus

IBV DILUTED 10^{-3} IN	DEAD/TOTAL	CUMULATIVE PER CENT SURVIVAL OF EMBRYOS INOCULATED						T_{70}
		Days after inoculation						
		1	2	3	4	5	6	
								days
Inactivated normal fluid...	28/28	100	64	0	0	0	0	3
Inactive virus...	20/29	100	97	93	62	48	31	>6

that a temperature of 56 C for 10 minutes did not destroy the activity of the interfering material.

Inasmuch as the simultaneous inoculation of inactive virus and active virus has been shown to result in a marked delay in the rate of embryo deaths (table 4), it was of interest to determine the effect of inoculation of inactive virus both before and after the inoculation of active virus. Accordingly, in the experiment described below 1.0-ml amounts of inactive virus were inoculated into the allantoic sac of a group of 30 embryos, similar amounts of tryptose broth being given another group of the same size. Thirty minutes later 0.2-ml amounts of IBV diluted 10^{-3} were inoculated into the allantoic sac of all embryos in both groups. The data obtained are presented graphically in figure 1. It will be

seen that all embryos receiving tryptose broth before the inoculation of IBV were dead on the second day after inoculation, whereas 68 per cent of the embryos receiving inactive virus before the inoculation of IBV were still alive on the sixth day after inoculation. It is clear from the data presented that when inactive virus was inoculated before active virus the rate of death of the embryos was strikingly delayed. A definite but not striking interference effect was obtained in the following experiment in which inactive virus was inoculated 3 hours after the inoculation of active IBV. Two groups of 40 chicken embryos were inoculated with 0.2 ml of IBV diluted 10^{-3}. Three hours later one group of embryos was inoculated with 1.0 ml of inactive virus and the remaining group was inoculated with the same volume of inactivated normal fluid. It was found that the T_{70} of the group receiving inactive virus was 5 days as compared with 3 days in the group of embryos receiving inactivated normal fluid. Thus it would
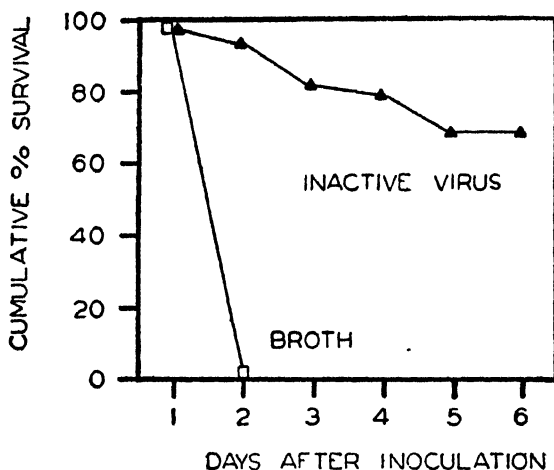
Figure 1. Protective effect of heated allantoic fluid containing interfering material on IBV.

seem from the experiments described above that interference was observed when inactive virus was inoculated before, together with, or after the inoculation of active IBV and that the degree of such interference was apparently greater when the inoculation of inactive virus preceded the inoculation of active virus.

DISCUSSION

It has been shown in the preceding sections that material capable of interfering *in vivo* with the multiplication of IBV in the chicken embryo was present in allantoic fluid from infected embryos stored for 24 hours at 36 C after death of the embryo but was not present in allantoic fluid from similarly treated normal embryos, infected embryos still living, or infected embryos dead less than 2 hours. Moreover, attempts to produce such interfering material by similar incubation of infected allantoic fluid *in vitro* alone or in the presence of normal

chorioallantoic membranes were unsuccessful. These findings would seem to indicate that the formation or liberation of interfering material following incubation of infected allantoic fluid for 24 hours at 36 C did not occur in the absence of surrounding (and possibly intact) infected tissue. It was also found that when undiluted allantoic fluid containing interfering material was heated for 10 minutes at 56 C it was rendered noninfective for chicken embryos but retained its capacity to interfere with the propagation of IBV. On the other hand, similarly heated allantoic fluid from living embryos (lacking in demonstrable interfering material) was still infective for 10 of 15 embryos. Thus it would seem plausible to suppose that a temperature of 56 C for 10 minutes served, primarily, to remove infective IBV from allantoic fluids containing interfering material rather than to produce such material in fluids in which no interfering material was readily demonstrable. Studies on the nature of the interfering material are planned.

SUMMARY

The rate of death of chicken embryos inoculated with undiluted allantoic fluid from infected eggs stored for 24 hours at 36 C after death of the embryo was found to be markedly slower than the rate of death of embryos inoculated with the same fluid diluted 1:10 or more. This phenomenon was not observed in titrations of infected allantoic fluid from living embryos or from embryos dead less than 2 hours and stored for 24 hours at 4 C. Attempts to produce such interference by similar treatment of normal embryos or by similar incubation of infected allantoic fluid *in vitro* alone or in the presence of normal chorioallantoic membranes were unsuccessful. Undiluted allantoic fluid containing interfering material and rendered noninfective for embryos by heat (56 C for 10 minutes) strikingly delayed the rate of embryo deaths when inoculated into the allantoic sac 30 minutes before active IBV.

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FACTORS AFFECTING THE BIOTIN CONTENT OF YEASTS[1]

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It has been known for some time that the content of certain vitamins in yeast can be varied by adding the vitamin or a precursor to the medium in which the yeast is grown. The uptake of vitamins by yeast cells has been utilized in the commercial production of so-called "enriched yeast." Thiamine and niacin are taken up avidly by *Saccharomyces cerevisiae* but riboflavin and pantothenic acid are not. The thiamine and niacin contents of yeast may be increased 110-fold for thiamine, according to Van Lanen *et al.* (1942), and 2- to 6-fold for niacin (Van Lanen, 1947; Peterson, 1948) by suitable additions to the medium. The response of *S. cerevisiae* to thiamine and niacin, on the one hand, and the lack of response to riboflavin and pantothenic acid, on the other, must be a reflection of the metabolic activities of the cell. Since thiamine pyrophosphate (cocarboxylase) plays such a prominent role in the decarboxylation of pyruvic acid in the alcohol fermentation and since production of ethyl alcohol and CO_2 are the major activities of the yeast cell, the abstraction of thiamine from the medium seems a logical response to these metabolic activities. This situation, however, does not exist with respect to niacin, which, although it is not an essential constituent of the medium, is readily taken up by the cells. Riboflavin is also not essential in the medium and if present is not taken up. Pantothenic acid is essential, but a surplus in the medium is not concentrated in the cells. The uptake of various vitamins does not seem to depend then upon the ability or inability of the cell to synthesize the compounds. It would be an attractive theory to explain the uptake on the basis of the physiological needs of the cell, but this cannot be done with any degree of assurance at the present time.

Added biotin is taken up in large quantities by lactic acid bacteria, e.g., *Lactobacillus pentosus* (Krueger and Peterson, 1948), but the response of yeasts, particularly yeasts other than the *Saccharomyces* types, has been investigated very little. In previous work from this university Massock and Baldwin (1943) reported that the biotin content of a number of bakers' yeast samples ranged from 0.6 to 1.8 μg per gram of dry weight and that the biotin added to the medium was taken up almost completely. It is the purpose of this paper to extend these limited observations to several types of yeast and to determine if possible the form of the biotin after it enters the cell, i.e., whether it exists in the free or the combined form.

EXPERIMENTAL PROCEDURE

Yeast strains. Transplants of *Candida albicans, Endomyces magnusii* 836, *Hansenula anomala* var. *spherica* 778, *Saccharomyces cerevisiae* 53, *S. cerevisiae* Y-30, *S. cerevisiae* "Gebrüder Mayer," *S. cerevisiae* "old process," *Torulopsis utilis* 2, *T. utilis* 3, *Torulopsis cremoris,* and *Willia anomala* were obtained from Dr. Elizabeth McCoy of the Department of Agricultural Bacteriology. Cultures of *Candida arborea, Candida pulcherrima, Oidium lactis,* and some other yeasts from Germany described as *"Lesser," "Mycelial,"* and *"Predominant"* were kindly sent to us by Mr. H. J. Bunker, Barclay, Perkins and Co., Ltd., London, and Dr. K. R. Butlin, Department of Scientific and Industrial Research, Chemical Research Laboratory, Teddington. Cultures of *Candida guilliermondii, Candida krusei, Candida zeylanoides, Debaryomyces globosus, Debaryomyces matruchoti, Endomycopsis fibuliger, Endomycopsis selenospora, Hansenula saturnas, Kloeckeri brevis, Mycoderma cerevisiae,* and *Pichia alcoholophila* were obtained through the courtesy of Dr. L. J. Wickerham of the Northern Regional Research Laboratory. A culture of *Rhodotorula gracilis* was kindly sent to us by Dr. Harry Lundin, Kungl. Tekniska Högskolan, Stockholm. There appear to be grave doubts among mycologists as to the validity of some of these names, e.g., *C. arborea, Lesser, Mycelial, Predominant,* but until the correct nomenclature has been established, we feel obliged to use the names given on the transplants at the time they were received.

Yeast transfers were made every month to an agar stab containing 1.5 per cent agar, 0.25 per cent glucose, and 0.25 per cent Difco yeast extract.

Media and fermentation. The inoculum medium contained 5.0 per cent beet molasses (Mason City), 2.0 per cent Difco malt extract, 0.75 per cent corn steep liquor, and 0.1 per cent $(NH_4)_2HPO_4$. It was prepared by adding a sterilized solution of $(NH_4)_2HPO_4$ to the clarified and sterilized solution of molasses, malt extract, and corn steep liquor at the time of inoculation. The fermentation medium containing Mason City molasses was prepared as described by Agarwal *et al.* (1947). Hawaiian molasses was clarified by means of corn steep liquor according to Agarwal *et al.* (1947). The medium was made up so as to contain 1 per cent reducing sugar, 0.1 per cent $(NH_4)_2HPO_4$, and 2 per cent of previously treated corn steep liquor (made by steaming a 1:3 diluted solution of crude corn steep liquor for 30 minutes, cooling, and filtering). The synthetic medium used for both the inoculum and fermentation was the same as that of Olson and Johnson (1949).

The media used for the experiments on biotin uptake were the same as the above, except that different levels of biotin were added. In case of the synthetic medium, biotin was omitted from the basal medium and added at different levels in the other fermentations.

In a 100-ml Erlenmeyer flask 15 ml of inoculum were grown by transferring the yeast from a stab culture to the sterilized medium and incubating on a reciprocating shaker (84 strokes, 10-cm length per minute) at 30 C for 24 to 36 hours. The yeast cells were removed by centrifuging, washed once with sterile water, and then made to the original volume. For the production of a yeast crop, 25 ml of fermentation medium were placed in a 500-ml Erlenmeyer flask

and inoculated with 1.25 ml of the inoculum suspension. The flasks were incubated at 30 C for 20 hours on a rotary shaker (made by B. F. Gump Company, Chicago) describing a 2.25-inch circle at 250 rpm. The yeast cells were separated from the medium by centrifugation and were washed with 25 ml of distilled water. The wash water was removed by centrifugation and combined with the first supernatant, and the mixture was used for the determination of unfermented sugar. The cells were suspended in distilled water and the volume was made up to 25 ml. This suspension was mixed very thoroughly just before two 10-ml portions were pipetted into test tubes for biotin assay and the determination of the yield of yeast. One of the 10-ml portions was centrifuged and dried to constant weight at 109 C.

Analytical methods. The yields of yeast, dry basis, were expressed as the percentage of sugar fermented. The micromethod of Shaffer and Somogyi (1933) (reagent no. 50 with 5 g KI per liter) was used for sugar determination.

All the biotin assays were based on the method of Wright and Skeggs (1944) except for that of soluble bound biotin. In order to improve growth and reduce the value of the blank the procedure was somewhat modified. Better growth in the inoculum medium was obtained by adding 0.5 ml each of salt solutions A and B (Krueger and Peterson, 1948) per 100 ml of medium. The casein hydrolyzate was treated with hydrogen peroxide to remove traces of biotin (Shull *et al.*, 1942). With these changes the blank was reduced to 0.6 ml of 0.1 N acid per tube. To facilitate the setting up of an assay, the tubes were placed in a copper rack and left unplugged. Each rack contained 50 tubes. A copper cover with a 2-inch side overhang, lined with a $\frac{1}{2}$-inch layer of cotton, was used in place of individual cotton plugs. After the addition of the test solutions and medium to the tubes, the rack was covered, autoclaved at 120 C for 15 minutes, and cooled. The rack was brought into the inoculation room, which had been steamed for 5 minutes. The tubes were inoculated and then incubated at 37 C for 72 hours.

(1) *Total biotin.* To a 10-ml portion of well-washed yeast cells 3.3 ml of 16 N H_2SO_4 were added, making the concentration 4 N. The tube was autoclaved at 120 C for 2 hours, neutralized, and diluted, and the biotin was determined. With each assay a sample of reference yeast was included as a check.

(2) *Free biotin and soluble bound biotin.* Another 10-ml portion of yeast cells was centrifuged and the supernatant liquid poured into a 125-ml Erlenmeyer flask. Ten ml of boiling distilled water were added to the cells and the contents were kept in a boiling water bath for 3 minutes. The hot water extract was separated by centrifugation and the extraction was repeated. The extracts were combined and assayed without further treatment for both forms of biotin. Free biotin is defined as the biotin found by assay with *Lactobacillus arabinosus.* This microorganism appears to be able to use only uncombined biotin, whereas *Lactobacillus casei* utilizes both free and soluble bound biotin (Bowden and Peterson, 1949). Soluble bound biotin is defined as the difference between the value found by *L. casei* assay and that given by *L. arabinosus.* The assay procedure with *L. casei* was according to Shull *et al.* (1942).

(3) *Insoluble bound biotin.* The residual cell material from the hot water

extraction was hydrolyzed with 3 ml of 4 N H_2SO_4, autoclaved, neutralized, and diluted, and the biotin content was determined.

(4) *Biotin in fermented medium.* Ten ml of supernatant and washings from the yeast cells were used for biotin assay without further treatment.

<div align="center">RESULTS</div>

Yields and biotin contents of yeasts. Yield and biotin content of yeasts grown on molasses media are given in table 1. Yields were in general higher in Hawaiian than in Mason City molasses. In some cases the difference was less than 5 per cent, which is not significant, but with eight of the yeasts differences of 5 per cent or more were observed. According to Agarwal and Peterson (1949) the nonsugar carbon in Hawaiian molasses, Mason City molasses, and corn steep liquor is 10.6, 6.7, and 10.7 per cent, respectively, of the total carbon. The amounts of fermentable sugar in the two kinds of molasses are almost the same. Since Hawaiian molasses contained 4 per cent more nonsugar carbon and was clarified with corn steep liquor, the higher yields in Hawaiian molasses are probably due to the utilization of more nonsugar carbon. For one yeast, *Rhodotorula gracilis*, a yield of 68 per cent was obtained in cane molasses medium and only poor growth in beet molasses medium. When the beet molasses medium was supplemented with synthetic medium, the growth was good, indicating that beet molasses was not toxic but lacks some growth substance required by *R. gracilis*. For *C. arborea*, *D. matruchoti*, and *H. anomala* up to 75 per cent yields were obtained. Such high yields must be attributed to the utilization of nonsugar carbon present in corn steep liquor and molasses. Although *W. anomala* utilized 95 per cent of the sugar, the yield nevertheless was very low, 17 per cent. Most of the sugar must have been converted into products other than cells. Five other yeasts, *K. brevis*, *P. alcoholophila*, *C. krusei*, *C. zeylanoides*, and *M. cerevisiae* (for which the data are not recorded in table 1), were grown, but the yields calculated on the sugar supplied were low, ranging from 11 to 21 per cent in Mason City molasses and from 23 to 41 per cent in Hawaiian molasses medium. Most of the sugar was left unfermented; only 20 per cent in Mason City and 40 per cent in Hawaiian molasses media were fermented. Even though the period of incubation was extended to 48 hours, not over 50 per cent of the sugar was utilized. Since two of these yeasts, *C. krusei* and *M. cerevisiae*, grew well in synthetic medium, it is probable that the beet molasses was deficient in required vitamin or nitrogen constituents. A sixth yeast, *E. selenospora*, did not grow at all in the molasses media unless a sulfur-containing amino acid, e.g., cystine or methionine, was added. This requirement is in agreement with Dr. Wickerham's results on synthetic medium, which he had kindly made available to us.

Olson and Johnson's synthetic medium gave good yields with some yeasts, viz., *S. cerevisiae* Y-30, *C. krusei*, and *M. cerevisiae*, but was not in general as good as molasses medium, probably because some of the nonsugar carbon in the latter was used for cell growth. The yields in Wickerham's medium (1946) were comparatively low (6.1 to 42.8 per cent). This medium was designed for classification purposes and not for maximum growth.

The biotin contents of yeasts in cane molasses medium were generally higher than those grown in beet molasses, since Hawaiian molasses had a higher biotin content (1.4 µg per gram) and the Mason City molasses contained only 0.1 µg per gram. *R. gracilis*, however, had a higher biotin content in Mason City molasses (on which its growth was poor) than in Hawaiian molasses medium. Apparently the little biotin present in the medium was concentrated in the small quantity of cells and thus gave a high biotin content per g of cells. Other yeasts also had high biotin content when the growth was poor. In Mason City molasses

TABLE 1

Yield and biotin content of different yeasts grown on molasses media

YEASTS	YIELD, % OF SUGAR FERMENTED		BIOTIN CONTENT, µg/g	
	Mason City molasses	Hawaiian molasses	Mason City molasses	Hawaiian molasses
Candida albicans	69.7	77	0.33	2.55
C. arborea	74.5	79.1	0.36	3.93
C. guilliermondii	62.8	73.1	0.31	2.7
C. pulcherrima	61.9	59.4	0.32	2.0
Debaryomyces globosus	41.8	48.2	0.9	4.3
D. matruchoti	73.5	76.7	0.23	2.1
Endomyces magnusii 836	67	68.7	0.24	1.85
Endomycopsis fibuliger	52.2	58.5	0.26	1.1
Hansenula anomala var. *spherica* 778	71.4	74.6	0.26	1.6
H. saturnas	55	58.5	0.34	2.4
Lesser 105	61.6	63.3	0.39	3.9
Mycelial	68.4	63.3	5.27	7.6
Oidium lactis	59	59.2	1.3	2.1
Rhodotorula gracilis	Poor growth	68.0	6.2	5.9
Saccharomyces cerevisiae 53	51.2	50.2	0.54	6.65
S. cerevisiae "Gebrüder Mayer"	47.7	54.1	0.46	3.9
S. cerevisiae "old process"	44.4	46.5	0.45	5.6
S. cerevisiae Y-30	48.6	49.1	0.5	4.95
Torulopsis cremoris	31.3	31.1	0.43	6.4
T. utilis 2	60.4	69	1.1	3.37
T. utilis 3	58.3	64.9	2.76	3.12
Willia anomala	15.2	17.2	2.7	7.5

medium the biotin contents of different yeasts varied greatly. For example, the biotin content of *E. magnusii* was only 0.24 µg per g whereas 5.27 µg per g were found in cells of the yeast designated *Mycelial*. This is more than could have come from the medium. Evidently this yeast synthesized biotin. Yeasts grown in the two molasses media usually had quite different biotin contents. For example, the figures for *C. arborea* in Mason City and Hawaiian molasses media were 0.36 and 3.93 µg per g, respectively. The biotin content of *T. utilis* 3, however, was not much increased by being grown in Hawaiian molasses medium. *T. utilis* apparently does not take up much biotin from the medium and depends largely on synthesis to meet its requirement. More data are

given in the next section regarding the biotin uptake by several yeasts. This experiment showed that some yeasts could take up biotin from the medium and others could synthesize it.

Uptake of added biotin from different media: Molasses media. Since the data in table 1 showed that yeasts grown in cane molasses contained more biotin than those grown in beet molasses and since the cane molasses contained about 14 times as much biotin as that of the beet, it seemed reasonable to attribute this higher content, in part at least, to uptake of biotin from the medium. Experiments were therefore set up to determine the ability of representative yeasts to take up biotin that had been added to the medium. The results with Mason City molasses medium are given in table 2. The biotin present in 100 ml of Mason City molasses medium (1 per cent sugar) was 0.2 μg. Additional amounts of biotin had no effect on the yield. The weight of cells given has been corrected for the weight of cells in the inoculum.

S. cerevisiae 53 requires biotin as a growth factor, but the yield of cells in the unsupplemented medium was as good as it was in the supplemented one. The former contained a small amount of biotin (0.2 μg per 100 ml), which was apparently sufficient to meet its requirements. Since this yeast cannot synthesize biotin, the biotin content of cells grown on the unsupplemented medium was low. This yeast took up large amounts of added biotin; the highest figure, 214 μg per g, was 400 times that of cells grown in unsupplemented medium, 0.54 μg per g. More than 85 per cent of the added biotin was taken up until the concentration in the medium reached 64 μg per 100 ml. Above this level, the uptake was much reduced and considerable amounts of biotin remained in the supernatant. When grown in unsupplemented medium the biotin content was low but most of it was present in insoluble bound form (83 per cent). With a high biotin content the free biotin amounted to over 95 per cent of the total. However, the insoluble bound biotin remained relatively constant—about 2.8 μg per g regardless of how much biotin was present in the cell. The recovery of added biotin in cells and supernatant was more than 90 per cent of that added, which shows that even at high concentrations of biotin this yeast did not destroy biotin during fermentation. Leonian and Lilly (1945) reported that the recovery of biotin was poor in fermentations with *Sordaria fimicola, S. cerevisiae* "old process," *Neurospora sitophila*, and *Ceratostomella pini*. In contrast, most of the yeasts tested in the present study gave a good recovery of added biotin.

T. utilis 3 synthesizes biotin. In unsupplemented medium containing only 0.2 μg of biotin per 100 ml, 1.8 μg were found in the cells after fermentation. Therefore, 1.6 μg of biotin were synthesized. On addition of biotin this yeast did not take up much of the compound. Most of it was not utilized and remained in the supernatant.

C. arborea does not synthesize biotin. It took up some biotin but much less than *S. cerevisiae*. There was very little increase in uptake when the medium contained more than 32 μg per 100 ml.

E. magnusii requires biotin but took up very little. After the biotin content reached 4.6 μg per g, very little more was taken up regardless of the amounts present in the medium.

The insoluble bound biotin of *T. utilis*, *C. arborea*, and *E. magnusii* was relatively constant at about 2 μg per g over a wide range of biotin additions.   Most of the biotin taken up was present in the free form.

TABLE 2

*Uptake of biotin by yeasts grown in beet molasses medium**

BIOTIN ADDED	WEIGHT OF DRY CELLS	BIOTIN IN CELLS		BIOTIN IN SUPERNATANT	RECOVERY OF ADDED BIOTIN	BIOTIN IN CELLS	
						Free	Insoluble
μg/100 ml	*mg/100 ml*	*μg/100 ml*	*μg/g dry cells*	*μg/100 ml*	*%*	*μg/g*	*μg/g*
			S. cerevisiae 53				
0	440	0.24	0.54	trace	—	0.07	0.45
8	446	5.28	11.8	1.58	86	8.95	2.91
32	470	28	55.2	3.2	91	52	2.77
64	381	52	136.7	6.0	90	131.6	2.89
128	476	68	142.8	57.2	98	134.4	2.65
256	450	96	214.0	162.4	101	190	2.8
			T. utilis 3				
0	670	1.8	2.76	0.03	—	0.012	1.04
8	687	3.0	4.36	4.2	90	0.9	2.04
32	650	3.5	5.4	26.4	93	1.4	1.8
64	711	3.4	4.8	57.6	95	1.04	2.1
128	638	3.2	5.0	102	82	1.22	2.35
256	600	3.0	5.0	234	93	1.83	2.33
			C. arborea				
0	670	0.26	0.36	trace	—	0.1	0.29
8	680	5.9	8.6	0.98	86	—	—
32	700	7.6	10.9	16.0	74	6.28	1.86
64	700	8	11.4	36.8	70	7	1.86
128	700	8.6	12.3	88.8	75	7.5	2.14
256	690	7.6	11	194	78	7.83	2.23
			E. magnusii 836				
0	747	0.165	0.22	0.003	—	0.003	0.2
8	752	2.4	3.2	4.32	84	0.98	1.73
32	744	2.96	3.98	26.4	92	1.36	1.78
64	727	3.36	4.62	56.4	93.5	1.85	1.96
128	728	3.65	5.05	124	99	1.54	1.86
256	752	3.6	4.79	245	97	1.78	1.56

* The average yields of *S. cerevisiae*, *T. utilis*, *C. arborea*, and *E. magnusii*, based on sugar fermented, were 43, 66, 69, and 66 per cent, respectively.

The free biotin and the insoluble biotin equal the total in *S. cerevisiae* cells but do not do so in the cells of *T. utilis* and *C. arborea*.   From 1.0 to 3.7 μg of biotin per gram of cells was not accounted for.   The probable explanation of this deficit is that the hot water extract of the cells contained some form of

biotin other than free biotin. Data to support this assumption is presented in table 5. The recoveries of added biotin were high (90 per cent) except in the case of *C. arborca*, with which it amounted to only 77 per cent. Perhaps there was some destruction of biotin by this yeast, but more data are needed for a definite conclusion.

TABLE 3

*Uptake of biotin by yeasts grown in Hawaiian molasses medium**

BIOTIN ADDED	WEIGHT OF DRY CELLS	BIOTIN IN CELLS		BIOTIN IN SU-PERNATANT	RECOVERY OF ADDED BIOTIN†	BIOTIN IN CELLS	
						Free	Insoluble
S. cerevisiae 53							
μg/100 ml	mg/100 ml	μg/100 ml	μg/g dry cells	μg/100 ml	%	μg/g	μg/g
0	376	2.5	6.65	1.0	—	3.45	2.53
8	334	8	24	2.26	95	—	3.0
16	336	13.4	40.2	2.5	85	—	2.97
32	356	27	75.8	6.0	95	—	3.38
64	320	40	125	16.0	84	119	3.43
96	348	52	149	36.4	89.5	149	2.87
128	370	48	130	64.0	86	136	3.51
T. utilis 3							
0	610	1.9	3.12	—	—	0.08	1.75
8	570	1.55	2.74	6.6	75.5	0.74	1.96
16	590	1.8	3.06	15.6	92	0.93	1.7
32	578	1.6	2.78	24.6	75	1.17	1.73
64	530	1.6	3.03	50.6	79	0.845	1.81
96	628	1.3	2.1	76.8	80.5	0.714	1.5
128	546	1.55	2.84	115.6	90	0.82	1.65
C. arborea							
0	496	1.95	3.93	0.17	—	2.77	—
8	516	4.86	9.41	3.26	75	6.37	1.71
16	460	4.88	10.6	8.84	73	10.2	1.41
32	468	6.0	12.8	21.6	79.3	12.6	1.4
64	522	8.62	16.52	50	87.7	14.5	1.1
96	480	9.2	19.1	80	90.3	14.6	1.56
128	532	7.76	15	100	82.4	14.1	1.13

* The average yields of *S. cerevisiae*, *T. utilis*, and *C. arborea*, based on sugar fermented, were 40, 65, and 60 per cent, respectively.

† One hundred ml of medium contained 2.8 μg biotin; this was counted in the calculation of recovery.

The uptake of biotin by yeasts grown in Hawaiian molasses medium is given in table 3. The uptake was in the same general range as for Mason City molasses medium. The biotin content of these yeasts in the unsupplemented medium was much higher, since this medium contains 2.8 μg of biotin per 100 ml.

Synthetic medium. The uptake of biotin by yeasts grown in synthetic medium is given in table 4. The composition of the medium was the same as that of

Olson and Johnson except for the omission of biotin. In order to limit the amount of biotin present in the inoculum only one drop of the washed inoculum was used.

S. cerevisiae 53, S. cerevisiae "Gebrüder Mayer," D. matruchoti, and E. magnusii all require biotin. The first two yeasts took biotin from the medium up to 43 µg per g. There was practically no increase in biotin uptake after the concentration in the medium reached 64 µg per 100 ml. E. magnusii took up very little biotin even in the presence of a high concentration in the medium. T. utilis and H. anomala grow without biotin and took up only a small amount from the medium. The recovery of added biotin with all of the foregoing yeasts, except E. magnusii, was over 90 per cent. This shows that there was no biotin destruction during fermentation. The amount of insoluble bound biotin was generally constant for each yeast regardless of the biotin content of the supplemented medium. The biotin taken up was mostly in the free form. S. cerevisiae 53 took up a great deal of biotin (42.2 µg per g), but much less than from beet or cane molasses. These results suggest that molasses contains some factors that promote the uptake of biotin by the cells.

Forms of biotin in enriched cells. In the discussion of table 2 it was noted that some biotin was missing in the hot water extract of T. utilis and C. arborea. It was assumed that there was some biotin other than the free form present in the hot water extract and that this biotin was not utilized by L. arabinosus. Bowden and Peterson (1949) found that L. casei could utilize both free and soluble bound biotin. In this experiment, therefore, L. casei as well as L. arabinosus was used for assaying the hot water extract. The results are given in table 5. The biotin uptake in this experiment was somewhat different from that reported in table 2, probably because the sugar concentration was higher and the fermentation period was shorter. Soluble bound biotin was calculated from the difference in biotin content of the hot water extract as determined by L. casei and L. arabinosus. Column 4 shows that the hot water extract of T. utilis contained more soluble bound biotin than free biotin. In contrast to the data in table 2, all of the biotin in the cells could be accounted for. However, in the case of C. arborea there was still some missing biotin. A possible explanation is that the hot water extract contained additional bound biotin which was not available to L. casei. All of the four yeasts contained some water-soluble bound biotin; the largest amounts were obtained from T. utilis and S. cerevisiae.

Extraction of biotin from living cells. In order to remove the biotin of the medium surrounding the centrifuged cells, the cells were washed twice and the second washing was analyzed for biotin to see if the number of washings was sufficient. It contained much more biotin than should have been present and suggested that washing and centrifuging removed biotin from the interior of the cells. Additional washing proved this assumption to be correct.

Cells that had been grown in the presence of 1 µg of biotin per ml were used in this experiment. In this medium the biotin taken up by the cells amounted to 7.2 µg per 10 ml (144 µg per g of dry cells), and 3.02 µg of biotin remained in the supernatant. In the cells, 7.06 µg were present in the free form and only 0.17

TABLE 4
Uptake of biotin by yeasts grown in synthetic medium*

BIOTIN ADDED	WEIGHT OF DRY CELLS	BIOTIN IN CELLS		BIOTIN IN SUPERNATANT	RECOVERY OF ADDED BIOTIN	BIOTIN IN CELLS	
						Free	Insoluble
$\mu g/100\ ml$	$mg/100\ ml$	$\mu g/100\ ml$	$\mu g/g\ dry\ cells$	$\mu g/100\ ml$	%	$\mu g/g$	$\mu g/g$
S. cerevisiae 53							
0	20	—	—	—	—	—	—
4	426	1.69	3.96	1.0	67	—	2.94
16	422	8.0	19.0	5.8	86	13.3	3.14
32	402	14.1	34.3	11.9	81	32.3	3.36
64	404	17.5	43.3	40.0	90	39.6	3.04
128	404	17.0	42.2	100.0	91	39.6	3.34
S. cerevisiae "Gebrüder Mayer"							
0	25	—	—	—	—	—	—
8	462	5.1	11.2	2.12	90	9.09	2.1
32	450	11.5	25.5	17.2	90	—	2.17
64	400	16.0	40.0	46.6	97	42.5	2.44
128	394	17.0	43.0	112.0	100	45.9	2.49
E. magnusii 836							
0	0	—	—	—	—	—	—
4	306	0.28	0.92	2.06	59	0.1	0.94
16	324	0.30	0.92	11.5	74	0.23	0.74
64	354	0.50	1.4	49.0	77	0.85	0.55
128	334	0.50	1.5	99.0	78	0.6	0.53
H. anomala var. spherica 778							
0	418	0.14	0.33	0.16	—	0.012	0.24
4	496	2.76	5.53	0.676	86	5.28	0.88
16	444	5.5	12.4	7.26	80	—	0.98
64	494	7.62	15.5	51.0	91	15.4	0.91
128	432	7.26	16.3	110.0	92	15	0.75
T. utilis 3							
0	531	1.4	2.63	0.6	—	0.9	1.64
8	546	2.12	3.89	5.15	91	2.65	1.56
32	552	2.92	5.29	29.4	100	3.99	1.54
64	532	5.34	10.1	60	101	6.77	1.51
128	543	6.7	12.3	126	103	6.35	1.52
256	558	7.18	12.9	252	102	8.96	1.57
D. matruchoti							
0	25	—	—	—	—	—	—
4	490	1.25	2.55	1.89	78	0.45	0.9
16	520	1.9	3.67	12	87	2.12	0.635
32	500	2.0	4.0	27	90	2.6	0.64
64	550	2.95	4.95	54	89	2.9	0.62
128	470	3.25	7.0	108	87	5.32	0.56

* The average yields of S. cerevisiae 53, S. cerevisiae "G.M.," E. magnusii, H. anomala, T. utilis, and D. matruchoti were 41.5, 42, 34, 45, 54.4, and 52 per cent, respectively.

μg were there as insoluble bound biotin. A 10-ml aliquot of the yeast culture was centrifuged and washed with 10 ml of distilled water each time. The washing was repeated 9 times under the same conditions of centrifugation. In the first washing 0.18 μg were found, which fact was probably due to some biotin left from the supernatant. The amount of biotin found in successive washings was quite uniform (0.043 to 0.05 μg). The total biotin removed by 8 washings was 0.386 μg, which is equivalent to 5.4 per cent of the total free biotin in the cells.

TABLE 5

The forms of biotin present in the cells
(Mason City molasses medium)

BIOTIN ADDED	DISTRIBUTION OF BIOTIN			
	Total	Free	Soluble bound	Insoluble
T. utilis 3				
μg/100 ml	μg/g	μg/g	μg/g	μg/g
0	3.46	0.025	0.29	2.95
8	5.1	0.59	0.84	3.39
32	6.04	1.18	2.29	3.13
64	7.4	1.56	2.62	2.86
128	7.63	1.9	2.02	2.91
S. cerevisiae 53				
35	25	19.6	2.6	3.45
H. anomala				
35	4.43	2.6	0.65	1.21
C. arborea				
35	7.22	2.77	1.56	1.32

The concentration of sugar in the medium was 1.88 per cent for *T. utilis*, and 1.74 per cent for *S. cerevisiae* 53, *H. anomala*, and *C. arborea*. The yields were 46, 38, 48, and 63 per cent, respectively, on sugar fermented—less than 0.1 per cent of sugar remained unfermented.

SUMMARY

Twenty-seven yeasts were grown in beet and cane molasses media for the determination of yield and biotin content. Seventeen of these gave yields (dry weight) of 50 per cent or more based on the sugar fermented. More than 90 per cent of the sugar in the medium was utilized. Five yeasts gave yields of 70 per cent or more. Five others grew poorly or gave yields of less than 20 per cent in beet molasses but grew much better in cane molasses medium. Yields of more than 50 per cent can be attributed to the utilization of nonsugar carbon in the molasses and corn steep liquor. Yields obtained from synthetic medium were in general below 50 per cent.

Biotin figures for the 22 yeasts ranged from 0.23 (*Debaryomyces matruchoti*) to 5.27 µg per g ("*Mycelial*") in Mason City molasses medium and from 1.1 (*Endomycopses fibuliger*) to 7.6 µg per g ("*Mycelial*") in Hawaiian molasses medium.

Uptake of added biotin in the media was studied for 7 of the yeasts. *Saccharomyces cerevisiae* 53, which requires biotin, took up as much as 214 µg per g in beet molasses medium but only 42.2 µg per g in synthetic medium. *Endomyces magnusii* also requires biotin but did not take up significant amounts from the medium. Biotin uptake of the yeasts, therefore, is not correlated with biotin requirement. *Torulopsis utilis* and *Hansenula anomala*, which synthesize biotin and hence do not require it, did not take up much biotin. The insoluble bound biotin for all of the 7 yeasts was relatively constant no matter how much biotin was contained in the cell. Most of the biotin taken up was in the free form. Some soluble bound biotin was extracted by hot water from all of the yeasts tested.

The free biotin was held rather loosely in the cells, as could be demonstrated by washing and centrifugation. Approximately 5 per cent of the total was removed by 8 washings and centrifugations. The form of the bound biotin is still unknown and is left for further work.

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ADAPTIVE ENZYMATIC PATTERNS IN THE BACTERIAL OXIDATION OF TRYPTOPHAN

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It has been shown recently that analysis of adaptive enzymatic patterns ("simultaneous adaptation") can be used to advantage in the unraveling of microbial metabolic reaction chains (Stanier, 1947). In essence, such analyses represent an extension and refinement of the customary kinetic approach to the problems of intermediary metabolism, made possible by the substrate-activated nature of many microbial enzymes. Simultaneous adaptation has been used to establish the nonparticipation of the tricarboxylic acid cycle in the oxidation of acetate by *Azotobacter agilis* (Karlsson and Barker, 1948), to study the mechanisms for the bacterial oxidation of aromatic compounds (Stanier, 1947, 1948), to examine the feasibility of suggested intermediates in nitrogen fixation (Burris and Wilson, 1946), and to investigate the utilization of uronic acids by *Escherichia coli* (Cohen, 1949).

The principle of simultaneous adaptation can be applied effectively only when it is possible to formulate a priori the various pathways that might be followed in an adaptively controlled metabolic process and to obtain a reasonable number of postulated intermediates for testing each formulation. These requirements at present limit severely the applicability of the method. After considering the various dissimilatory processes for which the above-mentioned limitations might not prove too restrictive, we concluded that the oxidation of tryptophan offered most promise. A good deal is now known about the degradation and synthesis of this amino acid, and many of the postulated intermediates in its metabolism were available to us. A study of the bacterial oxidation of tryptophan was, accordingly, undertaken.

MATERIALS AND METHODS

Through the kindness of Dr. S. H. Hutner we obtained several strains of bacteria, originally isolated by appropriate enrichment procedures, which were known to be capable of using the oxidation of tryptophan as the sole source of energy for aerobic growth. As a result of preliminary experiments on the adaptation to and oxidation of tryptophan by these organisms, one strain (Hutner, str. 7) was selected for detailed study and has been used throughout the experiments reported below. The characteristics of this organism, which will be designated here as *Pseudomonas* sp., will be described in a forthcoming publication by Dr. Hutner.

Stock cultures were maintained on yeast agar slants. In order to produce suspensions of cells with the desired enzymatic patterns for manometric work, the organism was grown on synthetic or semisynthetic media containing a single

compound as the source of carbon and energy. All these media had the following basal composition: NH_4NO_3 0.1 g, K_2HPO_4 0.1 g, $MgSO_4$ 0.05 g, agar 1.5 g, and distilled water 100 ml. The specific compound that served as a source of carbon and energy was added at a final concentration of 0.2 to 0.5 per cent, and the pH was adjusted to 7.0 to 7.2. So-called "unadapted" cells (i.e., cells not adapted to any compounds related to tryptophan) were produced by using asparagine as the carbon source. The organism grows sparsely on a strictly synthetic medium, and at times a small amount of yeast extract (0.01 per cent) was added to the basal medium in order to improve development. This addition has no effect on the adaptive patterns with which we were concerned, since the amount of tryptophan thereby introduced into the medium is very small. Even growth on a medium containing 0.5 per cent yeast extract results in only a partial activation of the tryptophan-oxidizing enzyme system. All cultures were incubated at 30 C.

Our supplies of certain compounds to which we wished to adapt *Pseudomonas* sp. were extremely limited. In such cases, specific adaptation was achieved by exposing suspensions of initially "unadapted" (asparagine-grown) cells to a small amount of the compound in question, rather than by growing them in its presence. As a rule, the activation of these resting cell suspensions was conducted in Warburg vessels with double side arms, one of which contained the activating substance. After the addition of this substance, the course of adaptation could be gauged from the oxygen uptake, and at the point when the activating substance had been completely metabolized, as judged by a return to the auto-respiratory rate of oxygen consumption, a second compound could be added from the other side arm.

All experiments were performed with the Warburg respirometer, at a temperature of 30 C in an atmosphere of air. Cell suspensions were prepared by harvesting the growth from plate cultures 18 to 24 hours old, washing once, and resuspending in M/60 phosphate buffer of pH 7.0. Carbon dioxide determinations were made by the "direct" method. Ammonia determinations were made on the supernatants from the cells used in Warburg experiments, Nessler's reagent being employed.

<div align="center">RESULTS</div>

General nature of the attack of L-tryptophan. The oxidation of L-tryptophan by *Pseudomonas* sp. is a strictly adaptive process. When cells grown on a medium containing asparagine as the sole source of carbon are tested manometrically for their ability to attack L-tryptophan, no oxygen consumption in excess of autorespiration occurs for 60 minutes following substrate addition, after which there is a typical exponential increase in the rate of oxygen uptake until a steady maximum rate is reached (figure 1). Cells grown with L-tryptophan as a carbon source show an immediate oxygen uptake when tested in a similar manner, and the rate remains steady to the point of substrate exhaustion. Cells grown on yeast extract attack L-tryptophan without a latent period, but the initial rate of oxidation is low and increases several times in the course of 2 to 3 hours (figure 1).

Presumably the amount of tryptophan in yeast extract is sufficient to cause partial adaptation.

When oxidizing limiting amounts of L-tryptophan, adapted cells consume approximately 145 microliters (6.5 micromoles) of oxygen per micromole of substrate, after which there is a return to a rate of oxygen uptake little, if any, greater than the autorespiratory one. The oxidation is accompanied by substantial carbon dioxide production, for which data are presented in table 1.
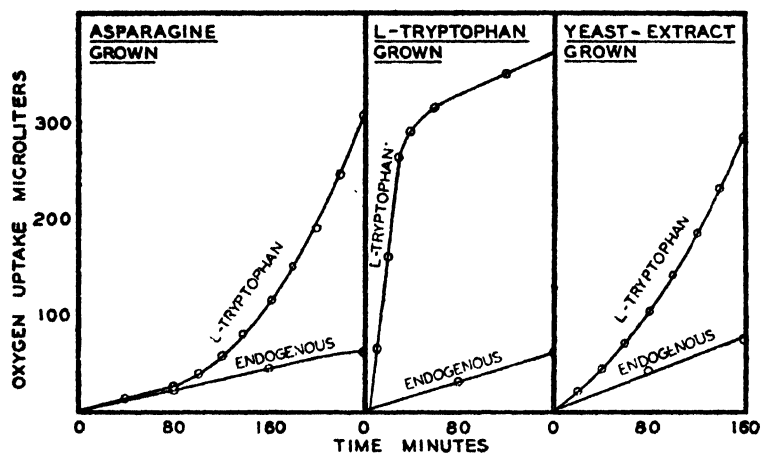
Figure 1. Effect of conditions of cultivation on the rate of oxidation of L-tryptophan (2 micromoles) by *Pseudomonas* sp.

TABLE 1

Oxidation of 3 micromoles of L-tryptophan by adapted cells of Pseudomonas sp.

TIME AFTER SUBSTRATE ADDITION	OXYGEN UPTAKE	CO₂ PRODUCTION	R.Q.
min	*microliters*	*microliters*	
15	109	101	0.93
30	252	246	0.98
60 (oxidation complete)	435	417	0.96

Oxygen uptake, microliters per micromole: 145.
CO₂ output, microliters per micromole: 139.
Average R.Q., microliters per micromole: 0.96.

The total carbon dioxide production is 139 microliters (approximately 6 micromoles) per micromole of tryptophan oxidized, and the R. Q. remains constant (within the limits of experimental error) throughout the oxidation. As shown by the data in table 2, ammonia is also liberated as a result of the oxidation, about 1.3 micromoles being produced per micromole of tryptophan decomposed. These facts demonstrate that the oxidation is a far-reaching one. On the other hand, the figures for oxygen uptake and ammonia and carbon dioxide production are considerably less than those required for a complete oxidation of the tryptophan molecule according to the equation:

$$C_{11}H_{12}O_2N_2 + 11.5\ O_2 \rightarrow 11CO_2 + 3H_2O + 2NH_3.$$
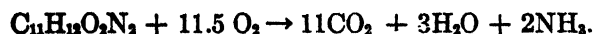
This discrepancy results, at least in part, from the occurrence of substantial oxidative assimilation. In the presence of 2,4-dinitrophenol (DNP) at concentrations between $M/4,000$ and $M/8,000$, the oxygen uptake per mole of tryptophan decomposed is substantially increased (see, e.g., figures 3 and 4), although even under these circumstances the theoretical uptake for complete oxidation has never been observed. The highest oxygen uptakes obtained in the presence of DNP have been approximately 80 per cent of the theoretical figure. The possibility thus exists that a small part of the tryptophan molecule is converted to organic end products not further attackable by the organism. We have not attempted to search for these hypothetical end products. Assuming that the discrepancies from the values for complete oxidation result exclusively from oxidative assimilation, the following equation best fits the data.

$$C_{11}H_{12}O_2N_2 + 6.5O_2 + 2H_2O \rightarrow 6CO_2 + 5(CH_2O) + 2NH_3,$$
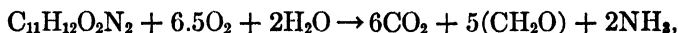
giving a theoretical R.Q. of 0.93 (found 0.96), a theoretical oxygen uptake of 145 microliters per micromole (found 143), and a theoretical carbon dioxide

TABLE 2

The production of ammonia from L-tryptophan by adapted cells of Pseudomonas sp.

TRYPTOPHAN OXIDIZED	NH₃-N FORMED	NH₃ PRODUCED	NH₃ PRODUCED
micromoles	*micrograms*	*micromoles**	*micromoles per micromole of tryptophan oxidized*
0.0	10.0		
1.0	27.0	1.21	1.2
1.3	31.5	1.55	1.2
2.67	61.5	3.70	1.4
4.0	77.0	4.80	1.2

* Corrected for blank value.

production of 134 microliters per micromole (found 139). The figures for ammonia production suggest that both nitrogen atoms are liberated as ammonia, part of the ammonia produced (about 35 per cent) then being assimilated and utilized by the cells.

Stereoisomerism and adaptive response. Figure 2 shows the oxygen uptake with different amounts of L- and of DL-tryptophan by cells grown in the presence of the L-isomer. The curves for the racemic mixture are those characteristic of a two-step oxidation with an intervening adaptive lag. The initial oxidation of DL-tryptophan proceeds at the characteristic rate for the oxidation of the pure L-isomer and ceases abruptly when the total oxygen uptake is exactly half that with an equivalent molarity of the pure L-isomer; this portion of the curve clearly represents the oxidation of the L-moiety. Following reversion to the autorespiratory rate, there is a slow and continuous increase in the rate of oxygen uptake until the total amount is double that at the initial break, when a second and permanent reversion to the autorespiratory rate occurs. We interpret the secondary rise in oxygen consumption as reflecting a much slower oxidation of

the D-moiety by means of a second, initially unadapted, enzyme system. Since the final total oxygen uptake per mole is identical for DL-tryptophan and for the L-isomer, it can also be inferred that the two enzyme systems oxidize tryptophan to the same extent.

In order to demonstrate more conclusively the initially unadapted condition of the D-tryptophan-oxidizing enzyme system in cells grown on L-tryptophan, use was made of the fact, originally discovered by Monod (1944), that DNP will completely block adaptation at molarities that do not affect dissimilatory mechanisms. The oxidation of L-, D-, and DL-tryptophan by cells adapted to the L-isomer was studied in the presence and absence of DNP (figure 3). In ac-
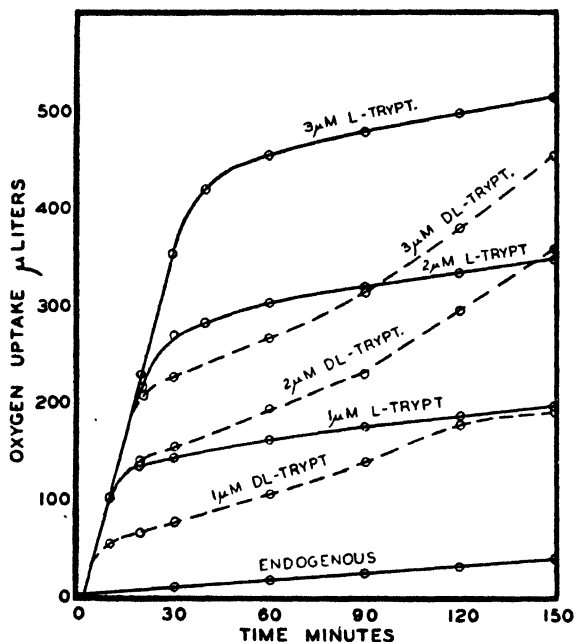
Figure 2. The oxidation of 1, 2, and 3 micromoles of L- and of DL-tryptophan by *Pseudomonas* sp. adapted to L-tryptophan.

cordance with expectation, treatment of such cells with M/4,000 DNP completely prevents attack on the D-isomer: the oxygen uptake with 2 micromoles of the racemic mixture is the same as with one micromole of the pure L-isomer, whereas with the pure D-isomer it remains permanently at the autorespiratory level. The same cell suspension without DNP oxidizes D-tryptophan after a typical adaptive lag and shows a secondary oxygen uptake with the racemic mixture of the type already described above.

If a similar experiment is performed with cells that have been grown on DL-tryptophan, entirely different results are obtained (figure 4). In such cells, both enzyme systems have been activated, and DNP is consequently unable to prevent oxidation of the D-isomer. Both in the presence and absence of DNP the L-isomer is oxidized somewhat more rapidly than the D-isomer, but the

difference is insufficient to cause more than a slight inflection in the later part of the curves for the oxidation of the racemic mixture. With all three substrates, the only effect of DNP is to increase somewhat the total oxygen uptake, a consequence of the blockage of assimilation.

In view of the striking specificity with which L-tryptophan activates and is acted upon by the L-tryptophan-oxidizing enzyme system, we anticipated that a relationship of similar specificity would obtain between the D-isomer and the enzyme system acting upon it. This proved not to be the case. Cells that have been adapted to the D-isomer alone are always simultaneously adapted to the L-isomer and actually oxidize the latter somewhat more rapidly than the former.
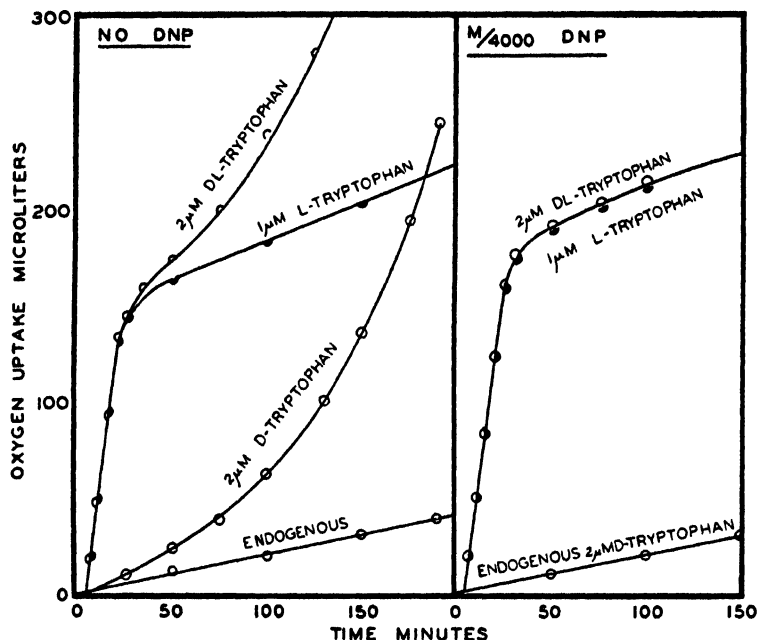
Figure 3. The oxidation of L-, D-, and DL-tryptophan in the presence and absence of DNP of *Pseudomonas* sp. adapted to L-tryptophan.

The adaptive response to D-tryptophan is thus effectively identical with the response to DL-tryptophan; the curves in figure 4 could represent both equally well.

It might be argued that the results obtained with the D-isomer were caused by the use of a preparation contaminated with a small amount of L-tryptophan. However, the data in figure 3 provide strong evidence against this possibility. If our D-tryptophan had contained any of the L-isomer, there should have been an appreciable, rapid oxygen uptake by cells adapted to the L-isomer in its presence; of this there is not the slightest indication. As a check on the possibility that an excess of D-isomer might inhibit the oxidation of a small amount of L-tryptophan by cells adapted to it, we studied the oxidation of a fixed amount of the L-isomer by L-tryptophan-adapted cells in the presence of various known

quantities of the D-isomer. Neither the rate nor the total quantity of oxygen consumed during the initial rapid oxidation of the L-isomer was affected, even by a tenfold excess of D-tryptophan. We feel that this possible source of experimental error is consequently eliminated.

Several interpretations of the dissimilar responses by *Pseudomonas* sp. to the isomers of tryptophan are possible, and we have been unable so far to obtain conclusive evidence as to which is correct. Considered from the standpoint of
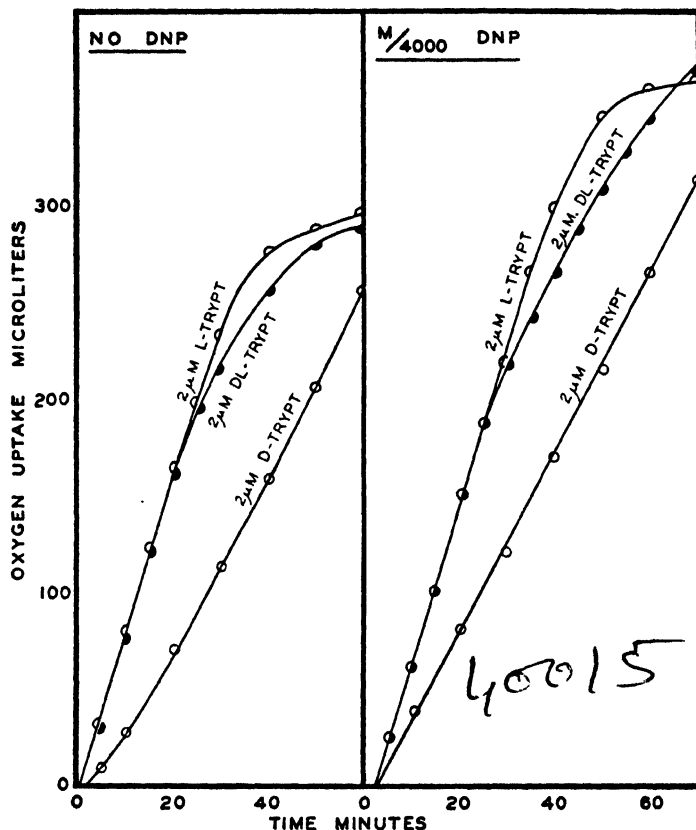
Figure 4. The oxidation of L-, D-, and DL-tryptophan in the presence and absence of DNP by *Pseudomonas* sp. adapted to both isomers by growth on DL-tryptophan.

adaptive response, the data could indicate that D-tryptophan activates nonspecifically the enzyme system attacking the L-isomer, as well as activating specifically the separate enzyme system attacking the D-isomer. Considered from the standpoint of *enzyme action,* however, the experiments are interpretable as indicating that the L-tryptophan-oxidizing enzyme system is specific in its action on the L-isomer, but the D-tryptophan-oxidizing enzyme system can act on both. Unless this dual action of the latter enzyme system were of a very special kind, though, exposure to L-tryptophan should cause simultaneous adaptation to the D-isomer, since it would provide a substrate activation equivalent to that

provided by the D-isomer for an enzyme system capable of action indifferently on both. Since this does not occur, an explanation in terms of enzyme action can be envisaged only if one assumes a *sequential relationship* between the D- and L-isomers; if the first step in the attack on the D-isomer involved the action of a racemase that transformed it to the L-isomer, the latter would in fact constitute an intermediate in the decomposition of the former, and the dissimilar adaptive responses would be explicable. Such an interpretation is compatible with the finding that any treatment that activates the D-tryptophan-oxidizing enzyme system causes an even greater rate of attack on the L-isomer, but at the same time this very fact precludes the possibility of demonstrating a racemizing action on D-tryptophan by living cells. Other observations concerning steric specificity, which are reported in the next section, tend to complicate further any attempt to interpret the foregoing results in terms of a racemizing enzyme. At present, therefore, the explanation of these findings remains obscure.

Evidence concerning the path of tryptophan oxidation. On the basis of what is at present known about the comparative biochemistry of tryptophan, a number of possible pathways can be postulated *a priori* for its oxidative degradation. These include:

(1) Reversal of the synthetic mechanism (Tatum and Bonner, 1944; Tatum, Bonner, and Beadle, 1944), involving a primary cleavage to indole and serine and a subsequent oxidation of the two fragments, anthranilic acid occurring as an intermediate in the further breakdown of indole.

(2) Rupture of the tryptophan molecule with the primary production of indole, ammonia, and pyruvic acid, the indole and pyruvic acid undergoing subsequent oxidation. The initial rupture is the mechanism for indole formation by *Escherichia coli*, as shown by Wood, Gunsalus, and Umbreit (1947).

(3) Primary attack on the aliphatic side chain, by oxidative deamination to indolepyruvic acid and subsequent oxidation through indoleacetic acid.

(4) Primary attack on the indole nucleus, with elimination of the second carbon atom in the five-membered ring, leading to the formation of kynurenin and thence, by oxidative deamination and ring closure, to kynurenic acid. This is the mechanism proposed by Kotake (1931, 1933) for tryptophan oxidation by the rabbit and by *Bacillus subtilis*.

In the light of the postulates concerning simultaneous adaptation (Stanier, 1947), one can predict specific adaptive patterns corresponding to each of these possible pathways in cells adapted to tryptophan. On the basis of (1), the cells should be simultaneously adapted to indole, serine, and anthranilic acid. On the basis of (2), they should be simultaneously adapted to indole and pyruvic acid, and possibly also to anthranilic acid. On the basis of (3), they should be simultaneously adapted to indolepyruvic and indoleacetic acids. On the basis of (4), they should be simultaneously adapted to kynurenin and kynurenic acid. The experiments performed to check these predictions have given extremely clear-cut results, which make it evident that none of the first three proposed pathways is operative and suggest strongly that the oxidation actually proceeds through the compounds in the fourth pathway.

Figure 5 presents the data on some of the intermediates that would occur in the first three pathways. The oxidation of indole and serine, either singly or in combination, is clearly far too slow in comparison to that of tryptophan for these compounds to act as intermediates. The same is true for indolepyruvic and indoleacetic acids. Data on anthranilic acid are given in figure 10, and show that it, too, cannot be considered a feasible intermediate.

On the other hand, kynurenin and kynurenic acid fulfill the postulated requirement for intermediates in tryptophan oxidation, since tryptophan-adapted cells are always simultaneously adapted to them and oxidize them at a rate comparable to the rate of tryptophan oxidation (figure 6). As shown by experiments with asparagine-grown cells, the oxidation of kynurenin and kynurenic acid is strictly adaptive, both substances being attacked in such experiments only after adaptive lags of the same order as the lag with tryptophan.

There are other experimentally verifiable consequences of the assumption that kynurenin and kynurenic acid participate in the reaction chain leading from tryptophan. In the first place, a fixed and calculable relationship should exist between the total oxygen uptakes with equimolar amounts of the three substrates, the figures for kynurenin and kynurenic acid being proportionately less than for tryptophan, in view of their more oxidized states. From the gross equations:
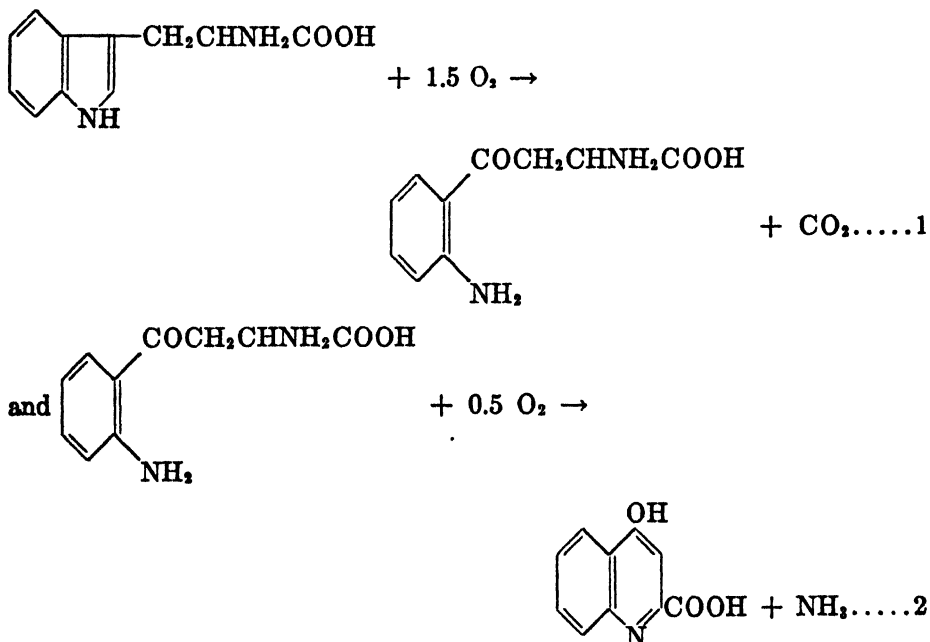
it is evident that the oxygen uptakes with kynurenin and kynurenic acid should be less by 1.5 and 2.0 moles per mole, respectively, than the oxygen uptake with tryptophan, assuming, of course, that the carbon atom eliminated from the indole nucleus appears as carbon dioxide, rather than undergoing assimilation.
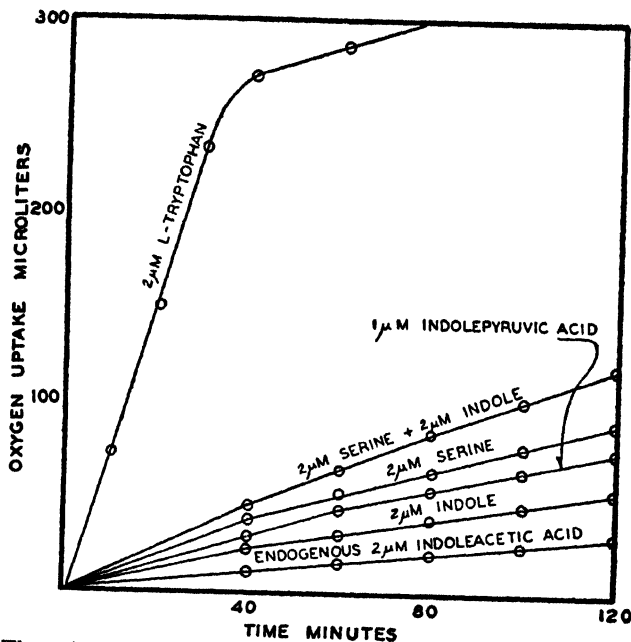
Figure 5. The oxidation of L-tryptophan and of various possible intermediates in its dissimilation by *Pseudomonas* sp. adapted to L-tryptophan.
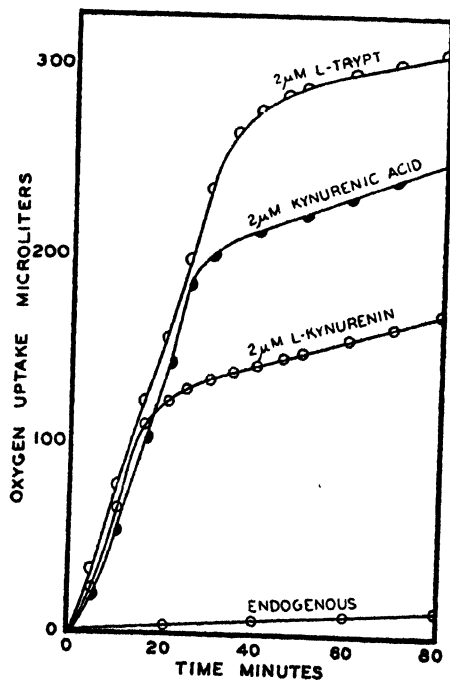
Figure 6. The oxidation of equimolar amounts (2 micromoles) of L-tryptophan, L-kynurenin, and kynurenic acid by *Pseudomonas* sp. adapted to L-tryptophan.

With kynurenic acid, the predicted oxygen uptake is always obtained, within the limits of experimental error (see, e.g., figure 6). Furthermore, the R.Q. is very close to the theoretical R.Q. derivable from the figures given previously for the oxygen consumption and carbon dioxide output with tryptophan. The data on the oxidation of L-tryptophan indicate an oxygen uptake of 6.5 moles per mole and a carbon dioxide production of 6.0 moles per mole, giving an R.Q. of slightly less than unity (0.93). Since the oxidation of tryptophan to kynurenic acid would result in the release of only one molecule of carbon dioxide, the R.Q. for the oxidation of kynurenic acid should, in consequence, be slightly greater than unity (5.0/4.5, or 1.11). In an actual experiment in which the R.Q. values were determined at three points during the oxidation of kynurenic acid, the figures obtained were 1.09, 1.18, and 1.14, which is in satisfactory agreement with expectation.

With kynurenin, however, the total oxygen uptake per mole is always far below the predicted level, being actually much lower (as shown in figure 6) than that with kynurenic acid. Our early experiments were performed with a sample of the natural L-isomer of kynurenin, and we assumed at first that the low total oxygen uptakes reflected gross impurity of the material. Subsequently, however, the experiments were repeated with synthetic DL-kynurenin, and oxygen uptakes of the same magnitude were obtained (allowing for the effects of stereoisomerism to be discussed below). It is consequently improbable that the anomalously low total oxygen uptakes with kynurenin result from the use of impure material, and we assume that the phenomenon is a real one. On the face of it, two explanations are possible. Kynurenin may not be involved at all as an intermediate in tryptophan oxidation, being broken down by an entirely different mechanism that results in a far lower total oxygen uptake per mole. But in this case, it is very difficult to understand how exposure to tryptophan can result in simultaneous adaptation to kynurenin (particularly in view of the carrying over of steric specificity in the adaptive response, described below), and how kynurenic acid, which meets so admirably all the requirements for an intermediate, can be fitted into a scheme for tryptophan degradation that does not involve also kynurenin. These difficulties can be circumvented by assuming that kynurenin is in fact an intermediate, but that there are two pathways for its metabolism, one (via kynurenic acid) resulting in a high oxygen uptake and the other unknown pathway resulting in a very low oxygen uptake. Furthermore, it is necessary to assume that when kynurenin is present in trace amounts (e.g., as an intermediate during the oxidation of tryptophan) it is nearly all decomposed via kynurenic acid, the other mechanism becoming substantially operative only when relatively large amounts of kynurenin are available.

If kynurenin is an intermediate in the oxidation of tryptophan, this particular sequence in the reaction chain would not involve the loss of asymmetry, since the side chain of the molecule that contains the asymmetric carbon atom is untouched. Hence one might predict that the stereoisomeric effects previously noted with tryptophan would carry over to kynurenin. Since both

L- and DL-kynurenin were available, this inference could be checked experimentally. As shown in figure 7, the attack on the stereoisomers of kynurenin is markedly affected by the specific adaptive patterns pre-established with respect to tryptophan. Cells that are adapted to both isomers of tryptophan are simultaneously adapted to both isomers of kynurenin, although (just as in the case of tryptophan) the oxidation of the D-isomer is somewhat slower than the oxidation of the L-isomer. On the other hand, cells that are adapted to L-tryptophan alone are likewise adapted to L-kynurenin alone, showing an oxygen
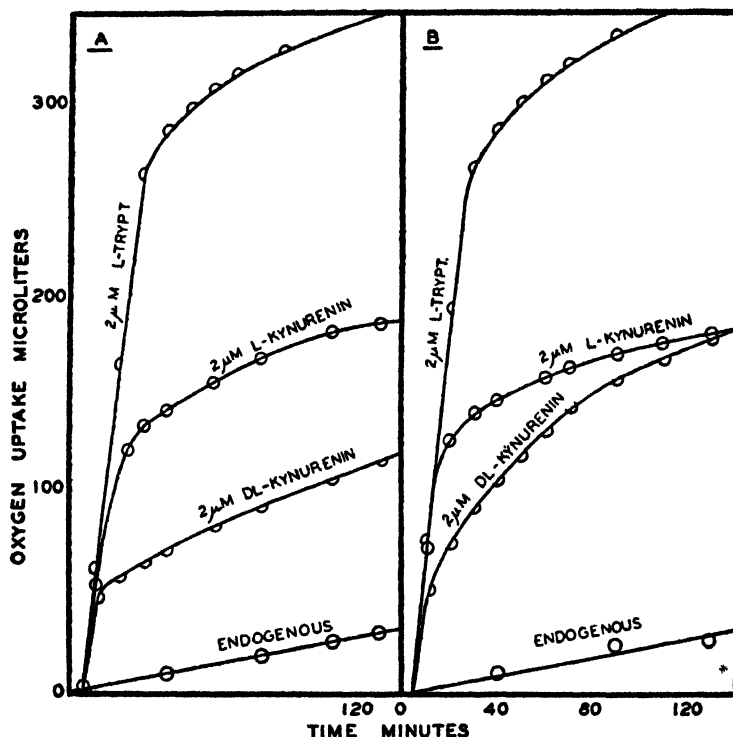
Figure 7. The effect of pre-established enzymatic specificity toward the stereoisomers of tryptophan on the oxidation of the stereoisomers of kynurenin by Pseudomonas sp. A, cells adapted to L-tryptophan; B, cells adapted to both L- and D-tryptophan.

consumption with DL-kynurenin that is only half the oxygen consumption with the L-isomer. One peculiar feature of this experiment deserves particular mention, since it provides indirect evidence for the postulated dual mechanism of kynurenin decomposition. When L-tryptophan-adapted cells act on DL-kynurenin, the total oxygen uptake remains *permanently* about half as great as with the equivalent molarity of pure L-isomer; no matter how long the experiment is continued after the initial rapid oxygen consumption has ceased, there is never any sign of a secondary adaptation to, and oxidation of, the D-isomer such as characterizes the attack on DL-tryptophan by similarly adapted cells. Inability to attack the D-isomer of kynurenin cannot, of course, provide an ex-

planation of the phenomenon, since cells adapted to DL-tryptophan clearly possess this ability. It suggests, rather, that the D-isomer disappears as a result of some other biochemical transformation, involving little or no oxygen uptake, during the time when adaptation to it would normally be taking place. Unfortunately, we did not have sufficient kynurenin to investigate this point further.

The adaptive patterns of cells specifically adapted to kynurenin and kynurenic acid are shown in figures 8 and 9. It will be seen that adaptation to kynurenic acid fails to bring about adaptation either to kynurenin or to tryptophan, whereas adaptation to kynurenin causes simultaneous adaptation not only to its presumed successor, kynurenic acid, but also to tryptophan.
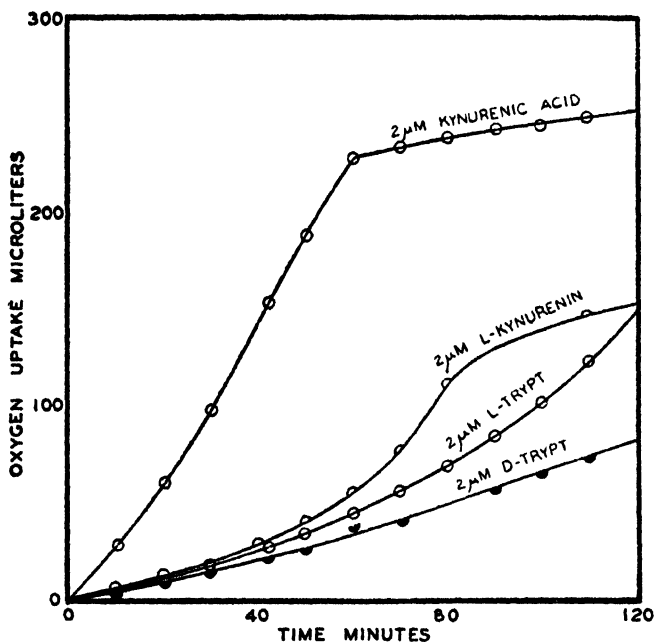
Figure 8. The adaptive patterns established in *Pseudomonas* sp. by preadaptation to kynurenic acid.

In addition to oxidation through kynurenin to kynurenic acid, a second pathway for tryptophan breakdown via kynurenin has been shown recently by the work of Beadle, Mitchell, and Nyc (1947) and Mitchell and Nyc (1948) on a mutant strain of *Neurospora crassa*. This involves an oxidation via kynurenin to 3-hydroxyanthranilic acid, from which nicotinic acid is formed by subsequent transformations of an unknown nature. Kynurenic acid does not participate in this reaction chain, the kynurenin being attacked presumably by substitution of a hydroxy group in the 3-position on the benzene ring. Although the evidence presented above pointed to the participation of kynurenic acid in the oxidation of tryptophan by *Pseudomonas* sp., it seemed desirable to check the possibility that the reaction sequence shown in *Neurospora* might be operative. Accord-
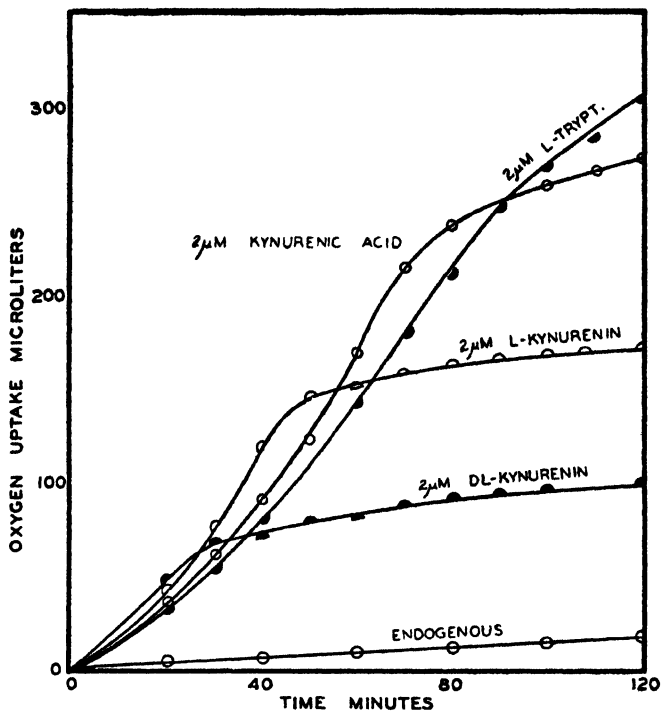
Figure 9. The adaptive patterns established in *Pseudomonas* sp. by preadaptation to L-kynurenin.
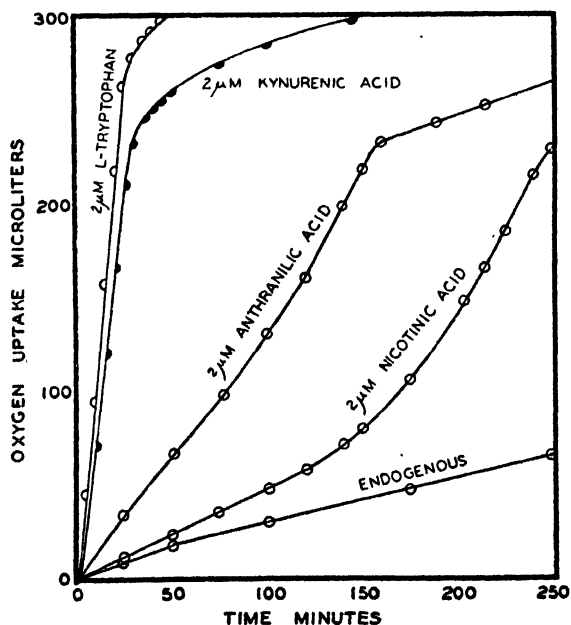
Figure 10. The oxidation of equimolar amounts (2 micromoles) of L-tryptophan, kynurenic acid, anthranilic acid, and nicotinic acid by *Pseudomonas* sp. adapted to L-tryptophan.

ingly, the adaptive response of tryptophan-adapted cells to nicotinic acid, anthranilic acid, and two hydroxy derivatives of the latter compound was determined. As shown in figure 10, *Pseudomonas* sp. can oxidize both nicotinic and anthranilic acids, but the low rates with these substrates after growth on tryptophan preclude the possibility that they participate in the dissimilation of tryptophan. The two derivatives of anthranilic acid (3-hydroxy- and 3,4-dihydroxyanthranilic acids) proved completely unoxidizable.

<div align="center">SUMMARY AND CONCLUSIONS</div>

Studies on the oxidation of D- and L-tryptophan by an unidentified organism, *Pseudomonas* sp., have provided evidence to suggest that the initial steps in the reaction occur as follows:

<div align="center">

D-tryptophan   L-tryptophan

↓   ↓

D-kynurenin   L-kynurenin

↘   ↗

kynurenic acid

↓

?

</div>
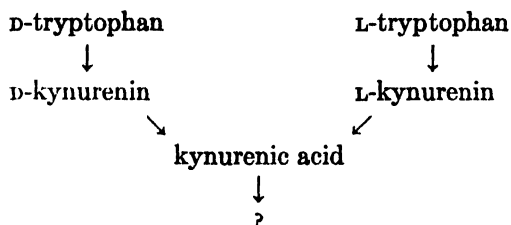
The participation of two enzyme systems in the initial attack on the two isomeric forms is clearly shown by experiments on adaptive specificity. The L-tryptophan-oxidizing system is specifically activated by, and specific in its action upon, the L-isomer, the D-isomer being oxidized only after a considerable adaptive lag by cells previously exposed to the L-isomer. The steric specificity in tryptophan oxidation is not absolute, however, since exposure to D-tryptophan produces cells capable of attacking both stereoisomers without a lag. The pattern of steric specificity set up by exposure to L-tryptophan carries over to kynurenin, such cells being unadapted to D-kynurenin, although they are simultaneously adapted to L-kynurenin.

The evidence for the participation of kynurenin in the foregoing scheme is weakened by the fact that the total oxygen uptake per mole with this substrate is always anomalously low in comparison to the oxygen uptakes with tryptophan and kynurenic acid. Since the oxygen uptakes with kynurenin were very similar irrespective of whether natural L-kynurenin or synthetic DL-kynurenin was used (allowing, of course, for the effects of stereoisomerism with the latter), it is improbable that the low oxygen consumption reflects the presence of impurities in the material used. A possible explanation, for which there is some indirect evidence, is that kynurenin can be broken down by another path involving a low oxygen uptake in addition to the path through kynurenic acid, and that the latter mechanism operates efficiently only when kynurenin is present in trace amounts, as would be the case during tryptophan oxidation.

The experiments on simultaneous adaptation show clearly that alternative possible mechanisms for the oxidation of tryptophan, such as a primary attack on the side chain or a rupture of the molecule with formation of indole, cannot be

operative.   By this means it was also possible to exclude the sequence through kynurenin to 3-hydroxyanthranilic and nicotinic acids.

The oxidation of tryptophan proceeds far beyond kynurenic acid, as shown by both oxygen uptake and carbon dioxide production, but the occurrence of oxidative assimilation makes it difficult to determine whether the reaction represents a complete combustion.   Although the oxygen uptake per mole of tryptophan is considerably increased by DNP, the figure even under these circumstances in our experiments was never greater than 80 per cent of that theoretically required for complete oxidation to carbon dioxide, ammonia, and water.

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A COMPARISON OF EIGHT ANTIBIOTIC AGENTS, IN VIVO AND IN VITRO[1]

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During the past year a number of new antibiotic agents have been studied by members of this department. Some of the observations, on separate agents, have already been published (Bliss *et al.*, 1948; Schoenbach *et al.*, 1948; Bryer *et al.*, 1948; Bliss and Chandler, 1948; Chandler and Bliss, 1948). In the present report, comparisons of the agents with respect to antibacterial activity, *in vitro* and in experimental infections in mice, are presented.

MATERIALS AND METHODS

Agents. Polymyxin D and aureomycin were received through the courtesy of The American Cyanamid Company and the Lederle Laboratories, Inc., during the fall of 1947. Polymyxin D, first described by Benedict and Langlykke (1947) and Stansly, Shepherd, and White (1947), is derived from filtrates of cultures of *Bacillus polymyxa*. The material used here is the hydrochloride, Lederle lot nos. 7-7795 and 7-8244.

Aureomycin is produced from *Streptomyces aureofaciens*. Its antibiotic properties were discovered by Dr. B. M. Duggar (1948) of the Lederle Laboratories and were first publicly described at a meeting in July, 1948. Lots 7-8020 A, 7-8071 A, 7-8254, and 7-8411 of the dried hydrochloride of this agent were used for the work that will be described. These lots were about 80 per cent pure aureomycin, according to a note from the manufacturers.

We are indebted to Burroughs Wellcome and Company for a supply of polymyxin B. The vials are labeled "Aerosporin-Brand." The history of the polymyxins is somewhat confusing. The one that was first described by Benedict and Langlykke and by Stansly, Shepherd, and White was derived, as mentioned above, from an organism identified as *B. polymyxa*. It is the one now known as polymyxin D. Almost simultaneously with its discovery, Ainsworth, Brown, and Brownlee (1947) announced that extracts of *Bacillus aerosporus* (Greer) had antibacterial activity. They named this product aerosporin but noted that *B. aerosporus* was called, by many investigators, *B. polymyxa*. Subsequently, Brownlee discovered that *B. aerosporus* (or *B. polymyxa*) produced at least two other agents besides the original polymyxin and aerosporin. This finding was reported at a conference of the Section on Biology of the New York Academy of Sciences held in May, 1948. Unfortunately the transactions of this meeting have not yet been published. After some discussion it was agreed that

Brownlee's three aerosporins should be called polymyxin A, B, and C, whereas the original polymyxin should be given the suffix D. According to a personal communication from Dr. Gladys Hobby, pure polymyxin B has 10,000 units per milligram and the hydrochloride, lot no. 0-896, studied here, has a potency of 7,050 units per milligram.

Sodium penicillin O (allylmercaptomethyl penicillin), research no. 8809, produced by The Upjohn Company, was received from the Antibiotics Study Section of the Public Health Service and was included in the study for comparison with penicillin G. A number of different samples of sodium crystalline penicillin G were used—Winthrop Chemical Company, lot 2326C, potency: 1,500+ u per mg, and Lederle lots 3811-421A and 3811-10,031, potencies: 1,532 and 1,533 u per mg. respectively.

A 2-gram sample of chloromycetin was kindly furnished by Dr. E. A. Sharp of Parke, Davis and Company, in October, 1948. This is labeled lot X3176. Chloromycetin, isolated from cultures of *Streptomyces venezuelae* by Burkholder of Yale and Ehrlich and his associates of Parke, Davis and Company (Ehrlich *et al.*, 1947), was shown to have antibacterial properties by the same investigators, and to be effective in certain rickettsial and viral infections by Smadel and Jackson (1947).

Circulin (Q-19) was received from The Upjohn Company in November, 1948. It was first described by Murray and Tetrault (1948), who reported that lyophilized preparations from a sporeforming, aerobic soil bacillus inhibited the growth of a number of gram-negative and a few gram-positive bacteria. According to these authors, the organism belonged to the same genus as *B. polymyxa* but was not identical with that bacillus; its cultural reactions most closely resembled those of *Bacillus circulans*. Independently, McLeod (1948) discovered another antibiotic from a strain of *B. circulans* and called it, likewise, circulin. In a footnote to her paper she stated that her agent differs from the one described by Murray and Tetrault in that the latter is "water-soluble, relatively nontoxic and more active against gram-negative than gram-positive organisms." The material studied here is the Murray and Tetrault product, and, to avoid confusion, it will be designated as Q-19 in the present paper. According to the manufacturers' statement there are 2,500 units of active material per milligram of drug in the first lot received—research no. 8836. Later a new lot was received, having double the potency of the first. It is designated as Q-19-1 in the tables.

The streptomycin used was E. R. Squibb and Sons' hydrochloride, control 11392.

Preparation of solutions. Except in the case of chloromycetin, the solutions for injection were made up in sterile distilled water. With chloromycetin the limit of solubility in water is about 2 mg per ml. When higher concentrations are required another solvent must be used. Smith *et al.* (1948) used 20 to 50 per cent propylene glycol but, since Prigal and his associates (1947) observed antibacterial substances in the blood of animals treated with 20 per cent propylene glycol, it was thought best here to use alcohol as the solvent for chloromycetin. With 20 per cent ethyl alcohol a 1 per cent solution could be prepared,

but even then crystallization occurred on standing.   For this reason solutions of chloromycetin, of over 0.1 per cent concentration, were left at room temperature and, if necessary, were warmed to 55 C before being used.   This may be done with impunity because as reported by Bartz (1948) and confirmed by us, the agent is stable to heat.   All of the other solutions were stored in a "deep freeze."

The agents were compared on a weight basis, penicillin, polymyxin B, Q-19, streptomycin, and chloromycetin invariably being weighed out.   Solutions of polymyxin D and aureomycin were usually so prepared, but this was not necessary as the vials had been filled on a weight basis rather than on a unitage or equivalence basis.

In vitro tests.   The basic medium used in the *in vitro* studies was Difco heart infusion broth to which 0.05 per cent of glucose was added.   One-half-ml volumes of serial twofold dilutions of the agents were inoculated with 0.5 ml of a 1:10,000 dilution of the test organism.   In the case of the cocci the final dilution was made in broth-containing 4 per cent of fresh, or washed, rabbit's red cells.   The tests were read after 18 to 20 hours' incubation at 37 C, and the presence or absence of visible turbidity or hemolysis was noted.

The organisms tested were stock laboratory strains and others recovered from patients during the year.   The authors are indebted to Miss Minnie Schreiber and Miss Rosemary Stokes of the Biological Division, The Johns Hopkins Hospital, for isolating a number of these organisms, and to Dr. Horace W. Smith for identifying the strains of *Proteus.*

Two or more agents were tested simultaneously, and polymyxin D or penicillin G were usually included as standards of comparison.   These agents, therefore, and aureomycin were the subjects of repeated trials.   The end points for a given organism and drug were not always the same, but, in order to simplify the tables, the one noted most frequently is shown as the minimal inhibitory concentration.

The effect of certain of the agents on the rate of multiplication of *Escherichia coli* or the C203 strain of beta hemolytic streptococcus was studied.   This was done by inoculating 10-ml volumes of drug-broth solutions with 0.1 ml of 1:100 dilutions of the cultures and plating out after 1, 3, 5, and 24 hours of incubation at 37 C.

In order to test the stability of the agents to heat and changes in the hydrogen ion concentration, a batch of heart infusion broth was divided into three lots, which were titrated to pH 5, 7, and 8 with hydrochloric acid or sodium hydroxide. To 25- to 30-ml aliquots at each pH sufficient antibiotic was added to make the desired concentration, namely, 200 μg per ml, for aureomycin, 100 μg per ml for streptomycin and chloromycetin, and 20 μg per ml for polymyxin D and Q-19. Each portion was subdivided in five 5-ml lots.   One of these was placed in the "deep freeze" for 24 hours; one was held at room temperature and another at 37 C for the same period; one, after 22 hours in the refrigerator, was placed in a 56 C water bath for 2 hours; the last, refrigerated for 23 hours, was heated in a boiling water bath for 10 minutes.   The solutions were then diluted out in broth, at pH 7.2, in twofold steps, using 0.5-ml volumes, and were inoculated with 0.5 ml of a 1:10,000 dilution of the test strain of *E. coli.*

Therapeutic tests.   White Swiss mice weighing 18 to 22 grams were used.   For

the first 3 months the mice came from a mixed stock; later, the purebred CF₁ mice were used.   Since the results with the two kinds of mice were quite different, they will be discussed separately.

TABLE 1

Effect of size of inoculum on titration end points

DRUG	E. COLI				HEMOLYTIC STREPTOCOCCUS, C203			
	Inoculum = 0.5 ml of dilution							
	1:5	1:500	1:50T	1:50M	1:5	1:500	1:50T	1:5M
	End points—μg/ml							
Penicillin G.......					0.1	0.012	0.012	0.006
Aureomycin.......					2.5	0.625	0.312	0.312
Chloromycetin.....	6.25	6.25	3.12	1.56	12.5	3.12	1.5	0.8
Streptomycin......	100	6.25	3.12	1.56	100	50	12.5	6.25
Polymyxin D......	10	0.625	0.15	0.04				
Polymyxin B......	5	1.25	0.625	0.15				
Q-19..............	10	2.5	1.25	0.625				

TABLE 2

Minimal inhibitory concentrations of antibiotics for gram-negative bacilli
(20-hour readings)

ORGANISM	AUREO.	CHLORO.	STREPTO.	Q-19	POLY. B	POLY. D
	Minimal inhibitory concentration, μg/ml					
E. coli, 4.................	5	10	6.25	0.625	0.312	0.156
E. coli, 9............	5	10	6.25	0.625	0.312	0.156
E. communior, 14.........	5	5	2.5	1.25	1.25	0.625
Citrobacter, 6............	5	5	5	1.25	0.625	0.312
Aerobacter, 10...	5	10	>100	1.25	2.5	0.625
Aerobacter, 12...	2.5	5	2.5	1.25	2.5	0.625
Aerobacter, 8..............	5	—	10	0.625	2.5	0.625
Aerobacter, 15.............	—	—	—	—	0.625	0.312
K. pneumoniae A (Lederle)	1.25	1.25	0.62	0.625	0.625	0.156
K. pneumoniae B (Cephus)	5	5	5	1.25	0.625	0.625
Proteus vulgaris, 11.......	6.25	2.5	5	>100	>100	>100
Proteus mirabilis, 18......	100	25	5	>100	>100	>100
Proteus mirabilis (Buck)..	100	6.25	25	>100	>100	>100
Proteus mirabilis (Herr)..	100	12.5	12.5	>100	>100	>100
Proteus unidentified, 17...	50	12.5	5	>100	>100	>100
P. aeruginosa (Butts).....	100	100	100	5	1.25	2.5
P. aeruginosa (Callo).....	100	>100	>100	1.25	2.5	2.5
P. aeruginosa (Herr)......	100	>100	25	2.5	1.25	1.25
P. aeruginosa, 16.........	100	50	50	5	1.25	1.25

Three organisms were used in the therapeutic comparisons: the C203 strain of hemolytic streptococcus, the SV1 strain of type I pneumococcus, received some years ago from Dr. Colin McLeod, and a strain of *Klebsiella pneumoniae* type A, received from the Lederle Laboratories and designated by them as KpnA-D.

Cultures were prepared by mouse passage the day before they were to be used. From the mouse they were transferred to blood broth and grown for 18 hours at 37 C.

The mice were infected by the intraperitoneal injection of 0.5 ml of a plain broth dilution of the culture. For C203 a 1:1,000 dilution was used, for SV1 the culture was diluted 1:5,000, and for *K. pneumoniae*, 1:500. These amounts resulted in inocula averaging 100,000 bacteria in the case of the first two organ-

TABLE 3

Minimal inhibitory concentrations of antibiotics for gram-positive cocci

(20-hour readings)

ORGANISM	AUREO.	CHLORO.	STREPTO.	PENI. O	PENI. G	Q-19
	Minimal inhibitory concentration, μg/ml					
Streptococcus group A, C203...............	0.31	5	12.5	0.008	0.008	100
Streptococcus group A, NY5...............	0.16	2.5	25	0.006	0.006	
Streptococcus group A, Cotton............	0.16	2.5	12.5	0.006	0.006	
Streptococcus group B, 090................	1.25	5	50	0.10	0.006	
Streptococcus group B, 19........	0.62	2.5	100	0.10	0.031	
Streptococcus group C, K61......	0.62	2.5	6.2	0.025	0.016	
Streptococcus group D, Zymog...	1.25	10	50	5.0	2.5	>100
Streptococcus group D, H69.......	0.62	5	50	—	2.5	
Streptococcus group D, 22A...............	0.62	10	50	5.0	2.5	
Streptococcus group F, For...............	0.62	2.5	6.2	0.10	0.05	
Streptococcus group F, H59............ ..	1.25	2.5	12.5	0.10	0.016	
Streptococcus group G, Dog.......... ...	1.25	2.5	12.5	0.10	0.012	
Streptococcus faecalis, Weston......... ..	1.25	10	50	5.0	2.5	
Streptococcus faecalis, Black.........	0.62	10	50	5.0	2.5	>100
Streptococcus faecalis, Tarr........	1.25	10	50	5.0	2.5	>100
Streptococcus faecalis, Twyman..........	0.62	5	10	5.0	2.5	
Streptococcus viridans, Dopkin...........	1.25	2.5	3.1	0.625	0.625	
Streptococcus viridans, Keel.........	0.62	10	12.5	5.0	2.5	
D. pneumoniae type I, SV1.......	0.31	2.5	12.5	0.016	0.016	>100
D. pneumoniae type I, Bailey............	0.12	1.2	2	0.025	0.012	>100
D. pneumoniae type III, Bayer.......... .	<0.15	2.5	12.5	0.025	0.025	
Staphylococcus albus, Heatly..............	0.62	5.0	2	0.062	0.016	>100
Staphylococcus aureus, Zeut..............	0.62	5	12.5	0.125	0.062	>100
Staphylococcus aureus, Zorn...............	0.62	5	2	0.125	0.062	>100
Staphylococcus aureus, Gelb..............	0.62	5	—	0.062	0.062	
Staphylococcus aureus, Gibb..............	0.62	10	—	0.062	0.031	

isms, and 600,000 in that of *K. pneumoniae*. The virulence of the specific culture was determined each time by injecting two mice each with three higher dilutions of the culture, from 1:1 million to 1:1 billion. These tests showed a variation in virulence that appeared to be unrelated to difference in the bacterial count. However, in most of the experiments with C203 and *K. pneumoniae* the infecting dose was approximately 10,000 MLD. The inoculum of 10^{-4} ml of pneumococcus culture also represented 10,000 MLD for the mixed breed of mice, but 100,000 for the pure CF_1 strain.

Immediately after each mouse in the treatment series was infected it was

given a subcutaneous injection of 0.2 ml of one of the drug dilutions. In most of the experiments two additional treatments were given, at 5½ and 23 hours after the infecting dose. Polymyxin B and D were compared at first on the basis of a single immediate treatment. The mice were observed for 7 days after the last treatment.

<div align="center">RESULTS</div>

Stability of agents. Chloromycetin was completely stable to heat at pH 5, 7, and 8. Even subjection to a temperature of 100 C for 2 hours did not raise the titration end point above that shown by solutions stored in the "deep freeze."
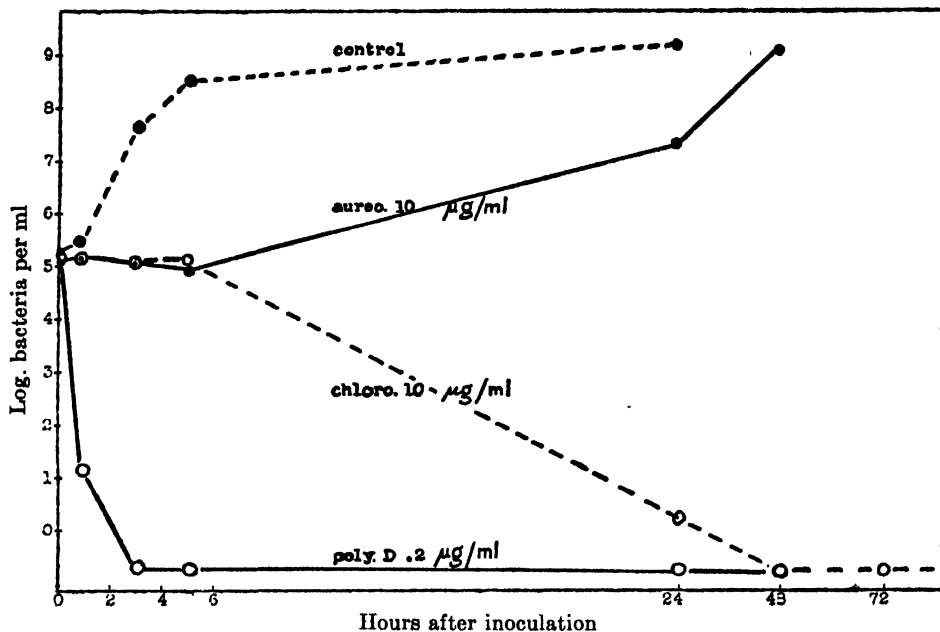
Figure 1. The effect of aureomycin, chloromycetin, and polymyxin D on the rate of multiplication of *E. coli.*

Streptomycin was less active when kept in an acid environment than when it was kept at pH 7, but polymyxin D and Q-19 were most stable at pH 5. All three of these agents were slightly affected by temperatures over 37 C. Aureomycin proved highly susceptible to heat, especially in the presence of alkali.

Effect of size of inoculum. Previous experience (Bliss *et al.*, 1948) showed that end-points with polymyxin D depended upon how many bacteria were inoculated. The other agents were tested in like manner, with the results shown in table 1. There it is seen that the behavior of all of the agents is affected by the size of the inoculum. Chloromycetin appears to be least subject to this factor, whereas polymyxin D is the most sensitive to it. For this reason, a standard inoculum of 0.5 ml. of a 1:10,000 dilution of the culture was used in all of the titrations, except those involving the strains of *Proteus.* These organisms had been found

so resistant to polymyxin D, in the earlier studies, that their cultures were diluted 1:100,000.

Minimal inhibitory concentrations. In tables 2 and 3 are shown the lowest concentrations of the agents which inhibited the growth of the various strains for

TABLE 4

Therapy of K. pneumoniae infection in mice, with polymyxin B and D, Q-19, streptomycin, aureomycin, and chloromycetin

(Strain KPnAD—type A)

	℞: SINGLE AT 0 HR					THREE, AT 0, 5½, AND 23 HOURS AFTER INFECTION						
	Mixed-strain mice					CF₁ mice						
Dose of drug per ℞	Poly. D	Poly. B	Poly. D	Aureo.	Chloro.	Poly. D	Poly. B	Q-19	Q-19-1	Strepto.	Aureo.	Chloro.
	Per cent survival (10–20 mice per dose)											
mg/kg												
80				80	90					100	90	85
70											90	100
50										100	70	65
35											70	80
25				10	10	100	100	100	90	100	20	0
10										100		
8				0	0	100		100	100	90		
4			80			90	100	100	100	60		
2.4						85	100	95	100	60		
1.6										40		
1.2			90			70						
1.0	70	85	30									
0.8						90	100	70	90	30		
0.6							60					
0.4	20	20	25			27.5	65	38	90			
0.3			0				20	0	80			
0.2	0	5	0			0	0	0	10			
0.1						0	0	0	0			
Median protective dose—mg/kg/treatment												
PD₅₀	0.8?	0.7?	1.0	58?	52?	0.7	0.4	0.5	0.3	2.3	38	42
95% confidence limits						0.5–0.95	0.33–0.53	0.35–0.81	0.18–0.51	1.6–3.2	30–46.8	37.6–47.0

20 hours. In general, the most effective agent against the gram-negative bacilli, *in vitro*, was polymyxin D. The least active was chloromycetin. This last, however, was more effective than either polymyxin D or aureomycin against the five strains of *Proteus*. These strains, all extremely resistant to the polymyxins and Q-19, varied in sensitiveness to aureomycin and were all moderately re-

sponsive to chloromycetin and streptomycin. Strains of *Pseudomonas* required high concentrations of aureomycin, chloromycetin, and streptomycin for inhibition.

Against the gram-positive cocci the two penicillins were generally most active; aureomycin occupied a middle position, and chloromycetin and streptomycin were the least effective of the agents in this class. Q-19 and polymyxin D (not shown in table 3) did not prevent the growth of any of the nine strains of cocci that were tested at concentrations under 100 μg per ml. It is to be noted that

TABLE 5

Therapy of hemolytic streptococcal infection in mice with penicillin G and O, aureomycin, chloromycetin, and streptomycin

(℞: 3, at 0, 5½, and 23 hours after infection. Strain: C203, group A)

DOSE OF DRUG PER ℞	MIXED-STRAIN MICE				CFi MICE				
	Peni. G	Peni. O	Aureo.	Chloro.	Peni. G	Peni. O	Aureo.	Chloro.	Strepto.
	Per cent survival (10–20 mice per dose)								
mg/kg									
100									70
80								0	43
50				33				10	30
25									10
10				0			85		
5				0			60		
3							30		
2			30				20		
1	100	100	0						
0.5	100	90	0		70	90			
0.4					73	60			
0.2	60	40			55	10			
0.16					0	10			
0.1	0								
0.06					10	0			
Median protective dose—mg/kg/treatment									
PD₅₀	0.18?	0.26?			0.22	0.33	4.3	>100	80
95% confidence limits					0.15–0.31	0.28–0.51	3.2–5.8		57–111.7

aureomycin inhibited the strains of *Streptococcus faecalis* and other enterococci at slightly lower concentrations than did either penicillin O or G.

Effect on the multiplication of bacteria. The effects of aureomycin, chloromycetin, and polymyxin D on the rate of multiplication of *E. coli* are illustrated in figure 1. Polymyxin D appears to be immediately effective in reducing the bacterial population and, in concentrations close to the minimal inhibitory concentration, in sterilizing the culture. Polymyxin B and Q-19 were also rapidly lethal in some of the tests, but their action was irregular and in general was not as striking as that of polymyxin D. Streptomycin, as well as chloromycetin,

had a slow but steady bactericidal effect. Aureomycin, at twice its minimal inhibitory concentration for *E. coli*, was only temporarily bacteriostatic.

With a strain of streptococcus as the test organism, penicillin G at 0.025 μg per ml and chloromycetin at 5 μg per ml were slowly bactericidal, whereas aureomycin at 1.25 μg per ml delayed growth only for 24 hours. Even at 5 μg per ml, a concentration equal to 10 times that required to suppress visible growth of the streptococcus for 24 hours, aureomycin failed to sterilize the cul-

TABLE 6

Therapy of pneumococcal infection in mice, with penicillin G and O, aureomycin, chloromycetin, and streptomycin

(℞: 3 subcutaneous injections at 0, 5½, and 23 hours after infection. Strain SV1, type I)

DOSE OF DRUG PER ℞	MIXED-STRAIN MICE		CF₁ MICE				
	Peni. G	Aureo.	Peni. G	Peni. O	Aureo.	Chloro.	Strepto.
	Per cent survival (10–40 mice per dose)						
mg/kg							
80						0	100
70							40
60			70	80			40
50						0	23.3
40			60	60			33.3
35							0
25	.		60	35	100	0	0
20		90					
10		40	30	3.3	90		
8		30			20		
6					20		
5	80		20	7.5			
4			10	10	0		
2.5			10	10			
2	60	10	10	5			
0.8	20		0	0			
Median protective dose—mg/kg/treatment							
PD₅₀	1.8	11.8	.22.5	30.5	8.0	>80	56.2
95% confidence limits	1.19–2.72	9.64–13.9	14.0–36.2	16.8–55.2	7.2–8.9		53.8–58.7

ture; and, although there were fewer than 10 organisms per ml at 24 hours, at 48 hours the count was 30 per ml, and by 96 hours full growth had occurred.

Therapeutic activity. The results of treating infections induced in mice with *K. pneumoniae* A, a type I pneumococcus, and the C203 strain of hemolytic streptococcus are shown in tables 4, 5, and 6. The percentage survival at the different doses is indicated, and also the median protective dose for each drug, as determined by the method of Litchfield and Fertig (1941). Where possible the 95 per cent confidence limits, for the PD₅₀, have also been given. Chloromycetin and aureomycin showed the same order of activity against the *K. pneumoniae* infection, whereas streptomycin, the two Q-19's, and the two polymyxins were

effective at much lower doses. Although the differences between the last four agents were slight, they were statistically significant (with P less than 4 per cent) in the case of polymyxin D and polymyxin B and of polymyxin D and Q-19-1.

Chloromycetin, used in concentrations up to the limit of its solubility in 20 per cent alcohol, had no effect upon the pneumococcal and but little upon the hemolytic streptococcal infection. Aureomycin was effective against both infections when three treatments with 10 to 20 mg per kg were used. The two penicillins protected against the streptococcal infection at the lowest dosage levels of any of the five agents tested, and penicillin G was superior to aureomycin in the pneumococcal infection in the mixed breed of mice. In the purebred mice, however, much larger amounts of penicillin than of aureomycin were required to protect 50 per cent of the mice against the pneumococci. This difference between the two kinds of mice, in amenability to therapy, was not noted in the other infections, nor with the other drugs. Moreover, the dosage-survival curves were much steeper in the case of the CF_1 mice infected with pneumococci and treated with either of the penicillins than they were in any other combination of circumstances. (An impression of the steepness of the dosage-survival curves may be had from a study of the spread of the data in the "per cent survival" columns in the tables.)

DISCUSSION

Attention was called in previous reports (loc. cit.) to the rapidly lethal effect of polymyxin D upon cultures of *E. coli* and to the transiency of aureomycin's action. The latter appeared to be the result of deterioration of the drug *in vitro*. When fresh aureomycin was added every 24 hours (Chandler and Bliss, 1948), growth could be prevented indefinitely, but, if not replenished, even amounts of aureomycin greatly in excess of the minimal inhibitory concentration failed to prevent eventual outgrowth. Chloromycetin behaves like aureomycin in the early stages of a growth study, but the decline in bacterial population is maintained and by 24 or 48 hours no viable organisms are found. In contrast to aureomycin, chloromycetin is stable to heat even in an alkaline environment.

One of the interesting aspects of the *in vitro* comparisons was the grouping of the compounds in relation to their effects on organisms belonging to the *Proteus* and *Pseudomonas* genera. The agents derived from *Streptomyces* were almost without effect on the *Psuedomonas* strains, but showed moderate activity for *Proteus*. With the bacterial derivatives the reverse was true. This pattern was adhered to by 10 additional strains of each organism with only two exceptions. Two strains of *Pseudomonas* were inhibited by less than 6 μg per ml of streptomycin. It is believed that the divergent responses of the two kinds of bacteria to the two kinds of agents must reflect differences in the metabolic processes of *Proteus* and *Pseudomonas* and in the modes of action of the agents.

Of the agents tested, streptomycin was the most unpredictable in effect on the gram-negative bacilli. All the way from 0.6 to 100 μg per ml of this agent was required for the inhibition of members of the coli-aerogenes group. Aureomycin, as noted also by Paine, Collins, and Finland (1948), varied in its effect on strains of *Proteus*. These authors found the minimal inhibitory concentration of

aureomycin for 13 strains of *Proteus vulgaris* to be over 100 μg per ml. The single strain of *P. vulgaris* shown in table 3 of the present report was considerably more susceptible than the three strains of *Proteus mirabilis*, but among the additional strains studied jointly with Dr. Horace W. Smith there appeared to be no association between species and sensitivity to aureomycin

Correlation between the *in vivo* and *in vitro* activity of the agents was good, but not perfect. In general, the order of effectiveness shown in the test tube was repeated in the animal experiments, but polymyxin D was effective *in vitro* at a lower concentration than any of the other agents, yet more of it was required for the successful therapy of *K. pneumoniae* infection in mice than of polymyxin B or the Q-19's. Moreover, there was a much greater difference between the therapeutic doses of aureomycin and streptomycin and of streptomycin and the polymyxin Q-19 group than was expected from the similarity of their minimal inhibitory concentrations for *K. pneumoniae in vitro*.

Chloromycetin, which *in vitro* seemed more active against the cocci than streptomycin, failed almost completely to protect mice infected with streptococci or pneumococci. The possibility that the doses of chloromycetin that were employed were approaching the toxic level was considered but was discarded in view of the relatively low toxicity of the agent for mice (Smith *et al.*, 1948). There is still the possibility that acute alcoholism may have contributed to the poor results with chloromycetin. Mice given 0.5-ml doses of 20 per cent alcohol by subcutaneous injection all survived but were quite ataxic for an hour. However, equally poor therapeutic results were obtained with a propylene glycol solution, administered by gavage in 50 mg per kg doses, to mice infected with streptococci. One can only conclude that chloromycetin is not effective in streptococcal and pneumococcal infections in mice at the doses to which we were limited by the low solubility of the agent.

Perfect agreement between the *in vivo* and *in vitro* orders of activity of a series of drugs is, of course, not expected because therapeutic properties are dependent upon the pharmacological behavior as well as upon the antibacterial activity of the agents. It would not have been surprising, therefore, had aureomycin, which is relatively slowly excreted (Dowling *et al.*, 1948), appeared more effective in the therapeutic trials than the rapidly excreted penicillins—and this was observed in the pneumococcal infection in the CF_1 mice. What is surprising is the difference between the results with penicillin in the two kinds of infection. The explanation presumably lies in the greater virulence shown by the pneumococcus for the CF_1 mouse than for the mixed-breed line. It seems possible that two treatments were enough for the mixed-breed mice, and that the third treatment, essential for the CF_1 mice, came too late to be of any avail in the case of penicillin. Whatever the cause of the difference, it stresses the danger incurred in drawing conclusions from experiments run in just one kind of animal.

SUMMARY

Most gram-negative bacilli were considerably more sensitive, *in vitro*, to polymyxin D and B and circulin (Q-19) than to aureomycin, chloromycetin, or streptomycin. The striking exception to this was the behavior of strains of *Proteus*

which, as a group, proved extremely resistant to the first three agents. The polymyxins and Q-19 were more effective than the other agents in the control of an experimental *Klebsiella pneumoniae* infection in mice.

Penicillin G and O inhibited the growth of most gram-positive cocci at lower concentrations than aureomycin, chloromycetin, or streptomycin, and were the most active agents in the treatment of an experimental hemolytic streptococcal infection. Although penicillin G protected one breed of mouse against a type I pneumococcal infection at low doses, in another breed both it and penicillin O were much less effective, ranking well after aureomycin.

Chloromycetin, which inhibited the growth of cocci *in vitro* at lower concentrations than streptomycin, was valueless as a therapeutic agent in the pneumococcal and streptococcal infections in mice.

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THE REVERSAL BY PABA OF SULFONAMIDE INHIBITION OF THE VIRUSES OF LYMPHOGRANULOMA VENEREUM AND MOUSE PNEUMONITIS[1]

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The antagonism of *para*-aminobenzoic acid (PABA) to the chemotherapeutic action of sulfonamides *in vitro* and *in vivo* has long been established by various workers (McCarty, 1941; Selbie, 1940; Thomas and Dingle, 1942; Woods, 1940). The studies of Woods and Fildes (Fildes, 1940; Woods, 1940; Woods and Fildes, 1940) provided, furthermore, a hypothetical picture of the metabolism of bacteria, based on the competitive antagonism of these two chemicals. The antagonism has also been extended to a sulfonamide-susceptible fungus by Dimond (1941) and malaria parasites by Seeler *et al.* (1943). The discovery of the chemotherapeutic action of sulfonamides on the lumphogranuloma venereum (LGV) virus (Felton *et al.*, 1943; Findlay, 1940a,b; MacCallum and Findlay, 1938; McKee *et al.*, 1942; Rodaniche, 1942) has led workers to investigate whether this antagonistic phenomenon of PABA and the sulfonamides could be applied to this sulfonamide-susceptible virus (Findlay, 1940; Rodaniche, 1942; Seeler *et al.*, 1943). However, the work was controversial. Findlay (1940), feeding 10 mg sulfanilamide and 10 mg PABA suspended in gum acacia to mice, found that PABA antagonized the chemotherapeutic action of sulfanilamide for the LGV virus injected intracerebrally. His finding was based on the development of symptoms and the death rate in mice. In contrast to Findlay's finding, Seeler *et al.* (1943), using a diet containing 0.3 per cent, 0.03 per cent sulfanilamide and 0.3 per cent PABA, respectively, in one experiment, and 0.03 per cent sulfamethyldiazine and 0.3 per cent PABA in another, found that PABA showed no antagonistic action in mice that had previously been infected by the intracerebral route with their strain of the LVG virus. Their finding was based on the percentage of survivors 28 days after the infection. Rodaniche (1943) fed a diet containing 2 per cent sulfathiazole and 0.5 per cent PABA to intracerebrally infected mice and found no evidence of reversal at this ratio. However, when he used an equal amount of the two chemicals together, he found that the PABA exerted some antagonistic action.

After the discovery that the 6BC strain of the psittacosis virus was susceptible to sulfadiazine (Early and Morgan, 1946a,b), Morgan (1948a,b) found that PABA definitely antagonized the chemotherapeutic action of sulfadiazine on this virus in eggs and that the chemotherapeutic action of the sulfadiazine was also reversed by pteroic acid and pteroylglutamic acid. He postulated that sulfadiazine prevented the growth of the 6BC strain of psittacosis virus by interfering with its use of the PABA in the synthesis of pteroylglutamic acid.

Since the psittacosis, lymphogranuloma venereum group of viruses have very close relationships, morphologically, developmentally, and tinctorially as well as antigenically (Rake et al., 1941), and many of the members are susceptible to the chemotherapeutic action of the sulfa drugs, a reinvestigation of the discrepancies just mentioned seemed justified, particularly because of the possibility of discovering biochemical differences among members of the group.

The introduction of the intranasal method for the study of the psittacosis, lymphogranuloma venereum group of viruses by the examination of the extensiveness of the lung lesions (Nigg and Eaton, 1944; Shaffer et al., 1940) furnishes a more quantitative method of measuring the degree of infection than does intracerebral inoculation. We applied this method to the study of the antagonism of PABA on the chemotherapeutic action of sulfonamides for the LGV virus in mice and extended this investigation to developing chick embryos. Because the mouse pneumonitis virus has been found to be susceptible to sulfonamides (Eaton and Hanford, 1945; Rake et al., 1942), similar experiments were done to see whether the same phenomenon occurred with this virus.

MATERIALS AND METHODS

Viruses. The J.H. strain of the virus of lymphogranuloma venereum and the Greb strain of the mouse pneumonitis viruses were used throughout the experiments. The mouse lungs were titrated after each passage for the production of stock virus and the 50 per cent maximum lesion score end point was determined (Horsfall, 1939; Reed and Muench, 1938) by killing the mice 6 days after inoculation and grading the lesions. The dilution corresponding to the 50 per cent maximum lesion score end point was used for infecting the mice in our experiments.

For the egg experiments the yolk sac passages of the LGV and the mouse pneumonitis viruses stored in this laboratory were used. Their LD_{50} had previously been determined by titration. A dose containing 10 to 100 LD_{50} was used in the experiments to be described.

Mice. Albino Swiss mice averaging 12 to 20 g in body weight were employed. They were bred in this department and rigid precautions have been taken against infection of any kind prior to use. In most of the experiments 12 mice were used for each dose of drug and 12 served as controls. The virus suspension was introduced intranasally in amounts of 0.05 ml, using light anaesthesia with ether. Drugs were administered intraperitoneally, first at 2 hours after inoculation with the virus and then daily for 6 days. Variations of this schedule will be found in certain experiments. Control mice received 0.5 ml of normal saline by the intraperitoneal route at the same time as the animals under treatment. Surviving mice were autopsied on the sixth day after inoculation. Mice dying before the fifth day were autopsied, and the extent of the lung lesions was recorded.

Drugs. Sulfadiazine (S.D.) was used in the majority of the experiments. Sulfanilamide (S.A.) was used only in a few cases for comparison. Sulfadiazine tablets were ground into fine powder in a mortar and suspended in normal physio-

logical saline. The volume was adjusted so that every 0.5 ml of the suspension would contain the required dose of the drug.

PABA was obtained from the Eastman Kodak Company. The sodium salt of this drug was employed instead of the acid itself.[3] To prepare the sodium salt, the powder was dissolved in sufficient N/1 NaOH to bring the pH to 7.6, with 0.2 per cent phenol red as the indicator. The final volume was adjusted by adding enough physiological saline so that 0.5 ml of the solution would contain the required amount of the PABA.

RELATION OF LUNG WEIGHT TO CONSOLIDATION AND TO BODY WEIGHT

In the experiments to be described the degree of pulmonary consolidation was determined both by gross examination and by weighing the lungs. The results of gross examination were recorded as pulmonary lesion scores according to the method of Horsfall (1939) with certain modifications: the minute discrete foci frequently seen with viruses of the psittacosis-lymphogranuloma group were recorded as a score of 0.5 when less than one-eighth of the total amount of lung tissue was involved. Otherwise the pulmonary lesion scores were determined as before, and the total score for a given group of animals was divided by 5 times the number of animals in the group to give a percentage score representing the degree of pulmonary involvement.

The weight of the lungs was determined, as an average, by weighing together the lungs of all the mice in a group. It was, of course, evident that several factors other than the extent of pulmonary involvement would affect the weight of the lungs. The most obvious were the age and weight of the mice, and attempts were made to minimize these factors by selecting mice so that all groups were of approximately the same average body weight and age at the start of the experiment. The average weights of each group were, however, different at the conclusion of the experiment after a period of 6 days. The mice treated with sulfadiazine continued to grow and to gain weight more rapidly than the controls. The untreated mice gained weight more slowly or lost weight because of illness from the infection. Maximum doses of PABA also tended to cause a loss of weight because of the toxicity of this substance at a dose of 40 mg per mouse, but smaller amounts were not toxic.

It was necessary, therefore, to assess the effects of dehydration, starvation, and cessation of growth on the weight of the lungs in normal mice and in those with pulmonary consolidation. Experiments with normal mice given toxic substances or subjected to dehydration and starvation revealed that no change of the lung weight occurred despite the loss of 20 to 35 per cent of the body weight as compared with control mice from the same group kept under normal conditions. It appeared from these results that in experiments in which loss of body weight

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[3] We found that when the same ratio of the acid and the sodium salt of PABA was used to a given amount of S.D. by weight, there was apparently no better antagonistic effect from the acid than from the sodium salt. Since the acid was more toxic and was more irritative to the peritoneum of the mouse when injected by this route, we therefore determined to use the sodium salt throughout our subsequent experiments.

occurred the original weight of the mice could be taken as representative of the lung weight, and it was assumed that loss of body weight would in most instances be accompanied by cessation of growth of the lung tissue.   In mice with consolidated lungs it is possible that dehydration might affect the weight of the lungs by reducing the amount of exudate, but data on this point are lacking at present.

When a gain of body weight occurred during the course of the experiment, this could be attributed largely to growth[4] and the resulting increase in lung weight over the controls might give misleading results unless some corrections were
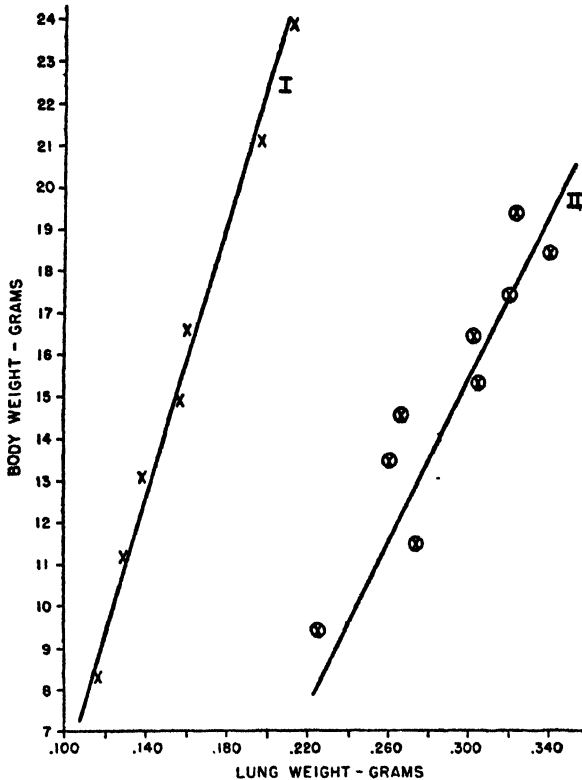
Figure 1. Relation of lung weight to body weight of mice. Line I, normal mice. Line II, mice with approximately 50 per cent of lung tissue consolidated.

applied.   Data were therefore obtained on the relation of lung weight to body weight by weighing the lungs of normal mice of body weights in the range between 8.5 and 24 grams.   The results as shown in figure 1 indicated a linear relationship between the two values.   The slope of line I (Fig. 1) for normal lungs gives a value for the rate of change in lung weight of 0.006 g per g of body weight. Data for the relation of lung weight to body weight of mice in which approximately 50 per cent of the parenchyma was consolidated were obtained from various experiments with the viruses used in the present work.   A summary of

[4] All mice were on a diet of dog "chows" and water ad lib.

the values for control mice receiving virus intranasally and saline intraperitoneally is shown in line II, figure 1. It will be seen from the position of this line that the lung weights, when about half the pulmonary tissue is involved, are almost twice that of normal lungs from mice of the same body weight. Here again the relationship appears to be linear, but the slope of the line is somewhat different from that of normal lungs and gives a value of 0.010-g increase in lung weight per g of body weight.

Since the mathematical relationships between lung weight, body weight, and degree of consolidation seemed rather complex, use of an approximate correction of lung weight based on the data in figure 1 seemed justified. A variable correction depending on the degree of consolidation was adopted as follows:

Lesion score of treated mice	Correction (K), g lung weight/g body weight
40–50	0.01
30–40	0.009
20–30	0.008
10–20	0.007
0–10	0.006

This correction was then applied as follows: $LW_T - (BW_T - BW_C)K = LW_T$ (corrected) in which BW_T and BW_C are body weights of treated and control mice, respectively, and LW_T is the lung weight of treated mice. In all instances the maximum body weights were used regardless of whether they were obtained at the beginning or the end of the experiment for the reasons given in a previous paragraph. The value of K depends on the lesion scores of the treated mice as noted above. Examples of this correction will be given in the next section.

DETERMINATION OF THE MINIMAL EFFECTIVE DOSE OF SULFADIAZINE

The minimal effective dose of various sulfonamides for the mouse pneumonitis virus has been determined (Eaton and Hanford, 1945), but for the purposes of the present experiments a more exact measurement was necessary to adjust the dose to a level such that the degree of pulmonary consolidation of the treated mice would be reduced to about 50 per cent of that of the controls. The results with the mouse pneumonitis virus are summarized in table 1. In mice receiving doses of 0.5, 0.1, and 0.05 mg sulfadiazine per day, the lesion scores and lung weights were significantly lower than in the control mice. The effects of treatment are also reflected in the gain of body weight in the animals receiving sulfadiazine as contrasted with the controls.

In the fifth column of table 1 the lung weights of the treated mice have been corrected to correspond to a body weight equal to that of the controls as described in the previous section. For example, in the first line the correction is $-(14.8 - 13.5) \times 0.006 = -0.0078$ grams, a relatively insignificant amount because the weight changes in all groups of mice were in the same direction. In the fourth line of the table, however, a much larger correction is required, in this case $-(14.6 - 11.8) \times 0.006 = -0.017$ or about 17 per cent of the average lung weight of the treated mice. Consideration of the data in table 1 will reveal some irregularities in the amount of reduction of lung weight of the treated mice in

relation to the dose of sulfadiazine, and the same is true of the lesion scores. It would appear therefore that small corrections in the lung weights would be overbalanced by other variables in the experiment. On the other hand, corrections of 10 per cent or greater may have some significance in the evaluation of the result, and their application seems justified.

The percentage of reduction in the lesion score and that in the lung weight in each case was calculated by the following formula: $(-1 \ T/C) \times 100$, in which T represents the percentage lesion score or the lung weight of the treated animals, and C represents those for the control. From the results in table 1 it will be seen that 0.05 mg S.D. gave more than 50 per cent reduction in the lesion scores and more than 30 per cent reduction in the lung weights, which we consider quite significant. The minimal effective dose of sulfadiazine for the mouse pneumoni-

TABLE 1

Determination of the minimal effective dose of sulfadiazine for the mouse pneumonitis virus

S.D.*	BODY WEIGHT		AVG LUNG WT.		L. W. REDUCTION	LESION SCORE	L. S. REDUCTION
	0 da	6 da	Observed	Corrected			
mg	*g*	*g*	*g*	*g*	%		%
0.5	11.2	14.8	0.140	0.132	−36	7	−80
0.1	11.2	15.0	0.147	0.137	−33	14	−60
—	11.8	13.5	0.205	—	—	35	—
0.1	12.1	14.6	0.102	0.085	−69	3	−91
—	11.8	11.3	0.210	—	—	35	—
0.05	12.5	14.5	0.168	0.162	−38	20	−60
—	13.7	11.5	0.261	—	—	50	—
0.05	14.5	18.3	0.187	0.161	−48	15	−66
—	14.6	14.3	0.309	—	—	44	—
0.01	19.3	18.9	0.335	0.327	+1	45	+15
—	21.2	18.0	0.325	—	—	39	—

* Daily dose of sulfadiazine per mouse.

tis virus was taken as 0.05 mg per day, since 0.01 mg had no effect on the lung lesions. Similar experiments in mice with the virus of lymphogranuloma venereum gave a minimal therapeutic dose slightly in excess of 0.01 mg, and for the sake of uniformity a dose of 0.05 mg was adopted with both viruses for the experiments on antagonism.

LACK OF THERAPEUTIC ACTION OF PABA

Experiments on the effectiveness of PABA against infection by these two viruses have been conducted in the same manner. The results of these experiments are presented in table 2. PABA did not protect the animals from these infections, even when as much as 10 mg was used (or approximately 0.7 mg per g of body weight). There is also no evidence from these or other experiments that PABA stimulated virus growth in the lungs of mice or in chick embryos. The data in the table also serve to indicate the degree of experimental variation to be

expected in the lung weights and in determination of the lesion scores. Thus changes in the lesion score of 20 per cent or less can be considered to have little significance, whereas changes in the lung weight of 12 per cent or less also lack significance under these experimental conditions.

THE ANTAGONISM OF PABA AND SULFADIAZINE

Mice of approximately the same body weight were divided into groups of twelve each and the drugs given to each group in conformity with the protocol in tables 3 and 4. As before, one group of the same number of mice was used for the control in each of the experiments. In addition, one group that received S.D. alone was run for comparison. Two hours after the intranasal introduction of the virus, a mixture of PABA and S.D. in 0.5 ml saline in the proportion indicated in the protocol for each experiment was injected intraperitoneally. The control

TABLE 2

Determination of the effect of PABA on the LGV and the mouse pneumonitis viruses in mice

VIRUS	EXPT	PABA*	RATIO OF LESION SCORES T/C†	PER CENT OF REDUCTION IN LESION SCORES	RATIO OF LUNG WT. T/C‡	PER CENT OF REDUCTION IN LUNG WT.
		mg				
LGV	I	10	42:47	−11	0.280:0.249	+12
	II	10	39:37	+5	0.287:0.273	+5
	III	10	37:40	−7	0.227:0.240	−1
Mouse pneum.	I	10	40:35	+14	0.264:0.243	+9
	II	10	46:39	+20	0.316:0.325	−3

* Daily dose of drug per mouse.
† *T*: percentage of lesion scores of the "treated" mice. *C*: percentage of lesion scores of the controls.
‡ *T*: Lung weight of the "treated" (corrected). *C*: Lung weight of the controls.

mice received 0.5 ml physiological saline, while the drug control received 0.05 mg S.D. in 0.5 ml saline. The injections were continued for six daily doses. Lung lesions, lung weights, and body weights were recorded as before, and the percentage of reduction of the lesion scores and that of the lung weights were calculated. Only the lung weights of the killed surviving mice were used for the calculation of the reduction in the lung weight, but the number of mice dying before the 6th day from the virus infection was negligible in the experiments recorded.

The results of these experiments are presented in tables 3 and 4. Here we found that in the case of the virus of lymphogranuloma venereum the chemotherapeutic action of the S.D. was completely antagonized by the PABA when the ratio by weight between the PABA and the S.D. used was raised as high as 800:1, according to the reduction in the lesion scores and that in the lung weights. There was some reversal, although not so marked when the ratio was 400:1. However, when the ratio was 200:1 to 100:1, there was no significant reversal.

TABLE 3

Antagonism of PABA on S.D. for lymphogranuloma venereum virus in mice

EXPT.	PABA	S. D.	RATIO OF PABA:S.D.	RATIO OF LESION SCORES T/C*	PER CENT OF REDUCTION IN LESION SCORES	RATIO OF LUNG WT. T/C†	PER CENT OF REDUCTION IN LUNG WT.
	mg	*mg*					
I	40	0.05	800:1	48:45	+7	0.227:0.235	−3
	20	0.05	400:1	27:45	−40	0.200:0.235	−17
	10	0.05	200:1	25:45	−44	0.196:0.235	−17
	5	0.05	100:1	22:45	−51	0.194:0.235	−17
	0	0.05	—	14:45	−69	0.186:0.235	−21
II	40	0.05	800:1	37:39	−4	0.187:0.200	−6
	20	0.05	400:1	27:39	−29	0.169:0.200	−15
	0	0.05	—	5:39	−87	0.151:0.200	−25
III	40	0.1	400:1	28:40	−28	0.178:0.228	−22
	20	0.1	200:1	18:40	−54	0.162:0.228	−30
	10	0.1	100:1	12:40	−69	0.163:0.228	−28
	0	0.1	—	9:40	−77	0.145:0.228	−36

* *T*: percentage of lesion scores of "treated." *C*: percentage of lesion scores of the controls.

† *T*: lung weights of the "treated." *C*: lung weights of the controls.

TABLE 4

Antagonism of PABA on S.D. for the mouse pneumonitis virus in mice

EXPT.	PABA	S. D.	RATIO OF PABA:S.D.	RATIO OF LESION SCORES T/C	PER CENT OF REDUCTION IN LESION SCORES	RATIO OF LUNG WT. T/C	PER CENT OF REDUCTION IN LUNG WT.
	mg	*mg*					
I	40	0.05	800:1	47:46	+2	0.309:0.312	+7
	20	0.05	400:1	40:46	−13	0.298:0.312	−5
	0	0.05	—	17:46	−63	0.182:0.312	−42
II	10	0.05	200:1	33:44	−25	0.234:0.307	−24
	5	0.05	100:1	26:44	−41	0.243:0.307	−24
	2	0.05	40:1	24:44	−46	0.191:0.307	−38
	0	0.05	—	15:44	−66	0.161:0.307	−48
III	20	0.05	400:1	28:33	−10	0.188:0.197	−5
	0	0.05	—	7:33	−77	0.155:0.197	−21
IV	40	0.1	400:1	33:32	+1	0.178:0.201	−12
	20	0.1	200:1	26:32	−18	0.182:0.201	−10
	10	0.1	100:1	28:32	−13	0.150:0.201	−21
	5	0.1	50:1	16:32	−49	0.136:0.201	−32
	0	0.1	—	3:32	−89	0.121:0.201	−40
V*	10	0.1	100:1	27:28	−3	0.198:0.208	−6
	10	0.5	20:1	24:28	−14	0.178:0.208	−14

* The PABA was given in divided doses.

We also found complete antagonism between these two drugs with the mouse pneumonitis virus when the PABA:S.D. ratio was raised to 800:1. However, with this virus there was a more conspicuous antagonism when the ratio was

400:1 than in the case of LGV (cf. experiments in tables 3! and 4). Furthermore, partial but definite reversal occurred at ratios of 200:1 and in some experiments at 100:1 with the mouse pneumonitis virus; this was not true of LGV.

The ratio between the antagonists in our experiments is much higher than those employed for the same phenomenon with bacteria and higher organisms. As a matter of fact, it may be partly due to the rapid excretion rate of the PABA *in vivo*, on one hand, and the higher sulfanilamide coefficient (Woods, 1942) of S.D., on the other. In order to verify the first point an experiment was conducted in which the daily dose of 10 mg PABA was divided into 6 doses given 3 hours apart. The first 5 doses of 1 mg each were given intramuscularly, but the last dose of 5 mg was given together with the S.D. intraperitoneally. Under these conditions greater continuity of the blood level of the PABA in the body of the mouse could be expected. The difference between the results of experiments II and V in table 4 clearly indicated that the rate of excretion of PABA played an important

TABLE 5

Antagonism of PABA on S.A. for the LGV and the mouse pneumonitis viruses in mice

EXPT.	VIRUS	PABA*	S. A.	RATIO OF PABA: S.A.	RATIO OF LESION SCORES T/C	PER CENT OF REDUCTION IN LESION SCORES	RATIO OF LUNG WT. T/C	PER CENT OF REDUCTION OF LUNG WT.
		mg	*mg*					
I	LGV	40	10	4:1	54:52	+4	0.301:0.306	−1
		10	10	1:1	18:52	−65	0.231:0.306	−25
		0	10	—	7:52	−87	0.178:0.306	−42
II	Mouse pneum.	40	10	4:1	58:48	+21	0.328:0.282	+16
		10	10	1:1	42:48	−12	0.280:0.282	−4
		0	10	—	20:48	−58	0.185:0.282	−34

* Total daily dose per mouse.

role in influencing the results. Under these conditions the reversal ratio was reduced to 100:1 and partial reversal occurred even at 20:1.

THE ANTAGONISM OF PABA AND SULFANILAMIDE

The sulfonamide used by the previous workers in experiments with the LGV virus was frequently sulfanilamide. Because of the fact that S.A. is less effective than S.D. for bacteria in terms of sulfanilamide coefficient, which is closely related to the amount of PABA needed to nullify the antibacterial effect of a given quantity of the sulfonamide (Woods, 1942), it seemed worth while to carry out experiments on antagonism between the PABA and S.A. with these viruses in order to find out whether the same phenomenon applied here. Previous studies had indicated that the minimal effective dose of sulfanilamide for these viruses was 10 mg per mouse per day (Eaton and Hanford, 1945), and this dose was used in the experiments on antagonism.

The results presented in table 5 were obtained from experiments similar to

those for the S.D. excepting that the mixture of the drugs was divided into two doses given 6 to 8 hours apart daily.   This was to maintain a longer contact between the two antagonists in the body of the mouse and to minimize the rapid rate of excretion of the PABA *in vivo*.   Complete antagonism of S.A. at a dose of 10 mg per day resulted when the PABA:S.A. ratio was 4:1.   There was no significant reversal when it was 1:1 in the case of the LGV virus, whereas almost complete reversal appeared at this same ratio in the case of the mouse pneumonitis virus.   The differing molecular weights of S.D. and S.A. do not appreciably affect these differences in the ratios.   A PABA:S.D. ratio of 400:1 in terms of weight is roughly 220:1 in terms of molecular weight, whereas the ratio of PABA:S.A. of 4:1 is 3.1:1 in terms of molecular weight.

EXPERIMENTS IN EGGS

Infection by the LGV and the mouse pneumonitis viruses in embryonic chicks through the yolk sac route was described some time ago (Rake *et al.*, 1940; Rake and Jones, 1942; Nigg and Eaton, 1944).   The therapeutic effect of sulfa drugs on these virus infections in eggs was also worked out (Eaton and Hanford, 1945; Jones *et al.*, 1941).   However, the antagonism of PABA on the chemotherapeutic action of sulfonamide for these two viruses in eggs through the yolk sac infection has not been determined so far as we know.   Further experiments were done by us to find the PABA:S.D. reversal ratio in infected eggs when these two viruses were used and to compare the results obtained with those for the mice, and with Morgan's (1948*b*) results with the 6BC strain of psittacosis.

Preliminary drug experiments showed that 2 mg of sulfadiazine was the minimal effective dose necessary to save 90 per cent or more of the infected eggs from the given dose of 10 to 100 LD_{50} of virus when compared with the controls, which received an equivalent amount of virus and physiological saline.   Four mg of PABA (sodium salt) was the maximum dose tolerated by the embryos.

Test of antagonism.   Three-tenths ml of the inoculum, which contained the desired dilution of either of the viruses diluted in nutrient broth, were introduced into the yolk sac of 7-day-old white Leghorn eggs, according to the usual method. Two hours later a mixture of the two drugs suspended in 0.3 ml saline, sterilized by being boiled in a water bath for 20 minutes, was introduced by the same route. The proportion of the drugs used is presented in tables 6 and 7.   Only one injection of the mixture was given.   Control eggs received an equivalent amount of sterile saline.   A drug control with 2 mg of S.D. alone was conducted in some of the experiments for comparison.   Eggs after inoculation were kept in the incubator at 36 C for 4 days.   At the end of the fourth day they were candled. ·Dead eggs were regarded as nonspecific deaths and not counted in the number of eggs tested.   The eggs were candled daily thereafter, until the eleventh day. From the fifth to the eleventh day dead eggs were recorded.   Survivors were sacrificed on the eleventh day.

· To prove that the death was due to the virus, the following procedure was used: Examination for bacterial contamination by direct smear from the yolk sac was made for each egg by- the gram stain.   Contaminated eggs were, of course,

disregarded. Yolk sacs of dead "treated" eggs were passed intranasally into mice to determine the extensiveness of the lung lesions produced. This was

TABLE 6

Antagonism of PABA on S.D. for the LGV virus in eggs

EXPT.	PABA	S.D.	SALINE	RATIO OF PABA: S D.	MORTALITY RATIO	PERCENTAGE OF DEATHS	PERCENTAGE OF REDUCTION
	mg	*mg*	*ml*				
I	0	2	0.3	—	0/6	0	−100
	0	0	0.3	—	6/6	100	—
II	4	2	0.3	2:1	6/6	100	0
	2	2	0.3	1:1	10/10	100	0
	1	2	0.3	1:2	3/4	75	−25
	0.5	2	0.3	1:4	7/9	77	−23
	0	0	0.3	—	11/11	100	—
III	0.2	2	0.3	1:10	9/11	81	−19
	0.1	2	0.3	1:20	2/6	33	−67
	0	0	0.3	—	9/9	100	—
IV	0.05	2	0.3	1:40	0/12	0	−100
	0.005	2	0.3	1:400	0/9	0	−100
	0	0	0.3	—	12/12	100	—
V	0.05	2	0.3	1:40	0/7	0	−100
	0.005	2	0.3	1:400	0/10	0	−100
	0	2	0.3	—	13/13	100	—

TABLE 7

Antagonism of PABA on S.D. for the mouse pneumonitis virus in egg

EXPT.	PABA	S D.	SALINE	RATIO OF PABA: S.D.	MORTALITY RATIO	PERCENTAGE OF DEATHS	PERCENTAGE OF REDUCTION
	mg	*mg*	*ml*				
I	0	2	0.3	—	0/5	0	−100
	4	2	0.3	2:1	3/4	75	−6
	2	2	0.3	1:1	7/8	87	+9
	0	0	0.3	—	8/10	80	—
II	1	2	0.3	1:2	7/8	87	−13
	0.5	2	0.3	1:4	6/9	67	−33
	0	0	0.3	—	12/12	100	—
III	0.2	2	0.3	1:10	9/12	75	−25
	0.1	2	0.3	1:20	7/12	58	−42
	0	0	0.3	—	11/11	100	—
IV	0.05	2	0.3	1:40	1/8	12	−86
	0.005	2	0.3	1:400	0/3	0	−100
	0	0	0.3	—	7/8	87	—
V	0.05	2	0.3	1:40	2/7	28	−64
	0.005	2	0.3	1:400	0/8	0	−100
	0	0	0.3	—	10/13	77	—

done by aseptically removing the yolk sac from each of the dead eggs immediately after candling and by grinding each separately in a mortar in the presence of

nutrient broth to make approximately a 10 per cent suspension. The suspension was centrifuged at 2,400 rpm and 0.05 ml of the middle layer of the centrifuged suspensions, which was relatively free of lipoid material, were injected intranasally into three mice. The mice thus subinoculated were observed for 7 days in the case of the LGV-virus-infected eggs and 14 days for the mouse pneumonitis. Mice dying within the observation period were autopsied and the extent of the lung lesions was recorded as before. The survivors were sacrificed at the end of the observation period. If significant lesions developed in the lungs of the mice, the embryo was counted as a specific death. Those which gave a lesion score less than 20 per cent were disregarded as nonspecific deaths. Yolk sacs of dead control eggs were not similarly tested. However, in our previous experiments, control eggs which died from the fifth day after the introduction of the virus produced with few exceptions extensive lung lesions when subinoculated into mice.

The mortality ratio, in which the numerator represents the number of dead embryos and the denominator the number tested, was used to indicate the results, and the percentage of reduction in the death rate between the treated and the control was compared by the formula $(-1 \ T/C)100$. The results of these experiments are presented in tables 6 and 7. From the data here it is clear that complete reversal of the chemotherapeutic action of the S.D. for both viruses began to appear only when 2 mg PABA and 2 mg S.D. were injected into the infected embryos. Partial but definite reversal occurred at ratios between 1:2 and 1:10. With the LGV virus no reversal at all occurred when the PABA:S.D. ratio was reduced to the range between 1:40 and 1:400. With the mouse pneumonitis virus there was some evidence of partial reversal in one experiment at a ratio of 1:40. The PABA:S.D. ratio, from which complete antagonism of the effect of the sulfonamide was achieved, was much smaller than that for mice.

DISCUSSION

As indicated by the present data, there is clear evidence that PABA did antagonize the chemotherapeutic action of sulfonamides for infections with the viruses of lymphogranuloma venereum and mouse pneumonitis in mice and in chick embryos. This confirms Findlay's work (1940) for the LGV virus in mice. The discrepancies in the previous work were presumably attributable to the rapid excretion rate of the PABA in mice; if the PABA is not given in adequate amounts, a complete reversal on the effect of the sulfonamide will not be attained, especially when the results are judged by mortality alone.

According to D. D. Woods (1940), the concentration of PABA required to overcome the chemotherapeutic action of sulfanilamide on the hemolytic streptococcus in vitro was 1/5,000 to 1/25,000, the concentration of sulfanilamide used. This is quite different from the same phenomenon with bacteria in mice. Selbie (1940), using hemolytic streptococcus for his experiment in mice, found complete reversal of the action of the sulfanilamide only when 25 mg PABA and 25 mg S.A. were used. McCarty (1941), testing the same phenomenon with pneumococcus type I in mice, found that complete reversal occurred only when

the total dose of 6 mg PABA and 24 mg sulfapyridine were used together. The ratio was 1:4. Thomas and Dingle (1942), performing the same experiment with meningococcus in mice and employing different methods of introduction of the drugs, found complete reversal of the effect of 0.005 mg S.D. when it was given together intraperitoneally with 1 mg PABA. The ratio was 200:1. Thus with bacteria the *in vivo* ratios for reversal were much larger than those for reversal of the sulfonamide activity *in vitro*.

It is quite possible that the rapid excretion of the PABA plays an important role in raising the ratio in mice. Martin and Rose (1945) found that the blood level of PABA following a single oral dose of 5 mg per 20-g mouse dropped from 12 mg per 100 ml within the first hour to almost nil in two hours, and that following a single subcutaneous dose of the same amount dropped from 20 mg per 100 ml within the first hour to zero in 3 hours. Although the determination of the amount of PABA recovered from the urine of the experimental mice is impractical, there is evidence to believe that a considerable amount, if not all, of the PABA must be excreted following the disappearance of the drug from the blood. Another factor is the relative rapidity of absorption of the PABA and S.D. from the peritoneum. The latter substance is relatively insoluble and therefore furnishes a depot in the peritoneum from which it is absorbed slowly, thus maintaining a rather persistent level of S.D. in the blood (Eaton and Hanford, 1945). The PABA on the other hand may be absorbed more rapidly, reaching an initial high blood level and then declining so that in intervening periods only the S.D. is present. This difficulty is partly overcome by giving repeated doses of PABA spaced at short intervals of time. The distribution of the drugs in various tissues and the amount of acetylated derivative formed *in vivo* varies with different sulfonamides as shown by Strauss *et al.* (1941), Peterson *et al.* (1941), and Shannon (1943). Similar differences in distribution and metabolic behavior between PABA and sulfadiazine may in part account for the high *in vivo* ratio.

Rapidity of excretion of the drugs has much less influence on the results with chick embryos. If we compare the data in tables 6 and 7 with the data of Morgan (1948b), who found that complete reversal of the chemotherapeutic action of S.D. on his 6BC strain of psittacosis virus occurred even when 0.005 mg PABA and 2.5 mg S.D. (ratio 1:500) were used whereas there was no reversal when the ratio was reduced to 1:5,000, we discover a remarkable difference between his data and ours, although his computations were somewhat different from the data presented here. The differences between these findings might be due to the differences in the viruses employed. In fact our results, particularly in mice, indicate a slightly higher reversal ratio for the agent of lymphogranuloma venereum than for the mouse pneumonitis virus both with sulfadiazine and sulfanilamide. The reversal ratio in eggs for these agents is at least 10 times as great as that found by Morgan for the 6BC strain of psittacosis.

Several observations indicate that the nature of the organism may be involved in altering the PABA:S.D. ratio. Dimond (1941) has found that the inhibitive action of sulfanilamide on *Trichophyton purpurum* was completely

nullified by PABA in a concentration as low as 1:500,000. This is very different from the concentration given by D. D. Woods (1940) for bacteria. On the other hand, Thomas and Dingle (1942) obtained a reversal of the effect of very small doses of S.D. on meningococcus when the PABA:S.D. ratio was 200:1, which is only one-fourth the ratio for the viruses we used in mice when the same drugs and the same route of administration of the drugs were employed. This finding is qualitatively in agreement with W. B. Wood's (1942) interpretation that the amount of PABA that nullified completely the antibacterial effect of a given quantity of the sulfonamide varied from organism to organism.

W. B. Woods also found that the ratio varied with a particular organism when different sulfonamides were used, and it has been found that the more effective the bacteriostatic power of the sulfonamide, the more PABA must be employed to give a complete reversal of its action. It has been found that sulfadiazine was 20 times more effective than sulfanilamide for bacteria if expressed in terms of the sulfanilamide coefficient. That sulfadiazine is at least a hundred times more effective than sulfanilamide was also found to be true in the treatment of mouse pneumonitis infection (Eaton and Hanford, 1945). The difference between the PABA:S.D. and the PABA:S.A. ratios observed in our experiment in mice conforms with this finding. In the first case we found complete reversal when the ratio was 800:1, whereas in the latter the ratio was 1:4, which is one two-hundredth of the other.

As the viruses have not been grown in lifeless medium, however, reservations must be made in speaking of a quantitative measurement of this phenomenon, because the absorption, the excretion, the distribution, and the acetylation of the drugs *in vivo* must all be taken into consideration. Our results supplement Morgan's observations in indicating that these intermediate agents, although apparently obligate intracellular parasites, are closely related to bacteria so far as the metabolism of *para*-aminobenzoic acid is concerned.

SUMMARY

Para-aminobenzoic acid reverses the action of minimal therapeutic doses of sulfonamides in mice infected by the intranasal route with the viruses of mouse pneumonitis or lymphogranuloma venereum.

The PABA:sulfadiazine reversal ratios were between 800:1 and 200:1 in mice. PABA:sulfanilamide reversal ratios were between 4:1 and 1:1. Slight differences in the reversal ratios for the two viruses were noted.

In chick embryos inoculated by the yolk sac route with either of these two viruses, the PABA:S.D. reversal ratios were between 1:2 and 1:10.

The findings are similar to the results of analogous *in vivo* studies with bacteria.

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A SEROLOGICAL STUDY OF SELECTED SPECIES OF ACTINOMYCETES

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Serological investigations involving organisms assignable to the families *Actinomycetaceae* and *Streptomycetaceae* as constituted at the present time (Breed *et al.*, 1948) have been conducted by other workers to a limited extent. The purpose of such studies has been primarily to demonstrate a relationship between some actinomycetelike organism and members of the family *Mycobacteriaceae* or, less frequently, of the family *Corynebacteriaceae* and the genus *Corynebacterium* in particular (Claypole, 1913; Goyal, 1937, 1938; Fritsche, 1908; Nelson and Henrici, 1922). Studies have also been made on the ability of certain actinomycetelike organisms to produce immunity or hypersensitivity in animals inoculated with the killed cells or cell extracts or in animals suffering active infections of these organisms (Bretey, 1933a,b; Drake and Henrici, 1943; Mathieson *et al.*, 1935; Nakayama, 1906). In all these studies the principal interest has centered about the pathogenicity of the organisms and the allergic response in the animal body. Attempts were made to relate such responses to like reactions produced in the animal body by the *Mycobacteriaceae*.

By serological methods Aoki (1935, 1936a) demonstrated what he has designated as three strains within a group of actinomycetelike organisms. On the basis of agglutination reactions he differentiated between an anaerobic strain, an aerobic diphtheroid strain, and an aerobic filamentous strain. The antigens for the test consisted of a saline suspension of organisms ground in a mortar. This method of preparation seemed satisfactory for the organisms in the first two groups, but presented difficulties in the third group because of the "thready" nature of the cells. In a later paper (Aoki, 1936b) he reported a procedure in which the antigenic material consisted of a suspension of "arthrospores" produced by the organisms. These structures were reported to be specifically agglutinable with serum antibodies. Although the identity of some of the organisms included in this study may be questioned, there is little doubt that at least some of them could be included among the actinomycetes at the present time. These reports are thus probably the first to describe agglutination methods applied to the actinomycetes and to demonstrate the difficulties to be overcome in such a study.

The studies reported in the present paper have as their purpose, first, the

development of serological methods applicable to members of the families *Actinomycetaceae* and *Streptomycetaceae* and, secondly, the evaluation of such methods as a possible aid in the identification of organisms within these families. In the light of the present greatly increased interest in the actinomycetes, such aids to identification would be most useful. Indeed, it is the opinion of the authors that serological methods would already have been more fully attempted were it not for the difficulties inherent in the organisms themselves, which make such methods, widely used for other microorganisms, entirely unsatisfactory. This fact would seem to explain the relative absence from the literature of studies on a serological approach to this problem. The difficulties encountered will be dealt with in subsequent sections of this paper.

EXPERIMENTAL PROCEDURES AND RESULTS

The species of organisms used in this study, as listed below, were chosen in order to include representatives of different groups of actinomycetes, the criterion of difference being the habitat from which the organisms may most commonly be isolated. Thus, included in the group are human and animal pathogens, a plant pathogen, and several saprophytic soil organisms. In the latter group at least two species were included that are known to produce antibiotic substances. The differences in the habitats of the various species were taken as possible reflections of differences in the physiological constitution of the organisms. If such fundamental differences were indeed present among the species, it seemed not unlikely that they might be indicative of differences in the serological reactivity of the organisms.

* *Actinomyces hominis* Waksman
    (*Actinomyces hominis* Bostroem. No. 3008)[3]
* *Actinomyces intermedius* (Krueger) Wollenweber
    (*Actinomyces intermedius* (Krueger) Wollenweber. No. 3329)
Nocardia asteroides (Eppinger) Blanchard
* *Nocardia farcinica* Trevisan
    (*Actinomyces farcinicus* Nocard. No. 3318
* *Nocardia madurae* (Vincent) Blanchard
    (*Actinomyces madurae* (Vincent) Lehmann and Neumann. No. 6245)
Streptomyces aureus (Waksman and Curtis) Waksman and Henrici
    (*Actinomyces aureus* Waksman and Curtis. No. 3309)
* *Streptomyces flavovirens* (Waksman) Waksman and Henrici
    (*Actinomyces flavovirens* Waksman. No. 3320)
Streptomyces griseus (Krainsky) Waksman and Henrici
Streptomyces lavendulae (Waksman and Curtis) Waksman and Henrici

[3] All organisms marked by an asterisk were received from the American Type Culture Collection, Washington, D. C. The names and numbers in parenthesis are those listed in the Catalogue of Cultures, 4th ed., 1938.

* *Streptomyces reticuli* (Waksman and Curtis) Waksman and Henrici
  (*Actinomyces reticuli* Waksman and Curtis. No. 3344)
* *Streptomyces scabies* (Thaxter) Waksman and Henrici
  (*Actinomyces scabies* (Thaxter) Guessow. No. 3352)

Streptomyces species[4]

Preparation of antigen for agglutination reaction. The growth from a 10-day nutrient broth culture, initial pH 7.2, containing beef extract 3 g, peptone 10 g, NaCl 5 g, and glycerol 20 g, in 1,000 ml of distilled water, was repeatedly washed and centrifuged in 0.85 per cent NaCl solution. Ordinarily the growth from one liter of culture medium provided a sufficient amount of material for the production of a concentrated antigen suspension. Maximum growth could be obtained by distributing the culture medium in 50-ml quantities in 250-ml flasks and incubating at room temperature in the dark.

After final washing the harvested cells were suspended in 30 ml of an 0.85 per cent NaCl solution, to which one part of merthiolate in 100,000 parts of suspension had been added as a preserving agent. Such a suspension was subjected to sonic vibrations for 30 to 40 minutes.[5] This treatment produced complete disintegration of the cells. The suspension showed an even turbidity and varied in color from white to gray, yellow, or lavender, depending upon the species of organisms involved. Microscopic examination of samples of such a suspension, taken at 5-minute intervals during the disintegrating process, showed a progressive breaking up of the filaments into very small fragments. For use in the agglutination tests the concentrated antigen suspension was diluted with 0.85 per cent NaCl to a standard turbidity corresponding to the no. 1 tube of the MacFarland scale.

Preparation of antigen for precipitin reaction. Samples of the suspensions of sonically treated cells were subjected to centrifugation at 3,000 rpm for 30 minutes. The suspensions to be so treated were placed in narrow glass tubes made by closing one end of a 6-inch length of glass tubing of approximately 4-mm bore.

After centrifugation the suspensions in the tubes had separated into three distinct zones. A small amount of sediment had collected at the bottom of the tube, representing the solid particles present in the suspension. Approximately half of the liquid column in the tube showed a distinct opalescence, but the upper portion of the tube contained a completely clear liquid fraction. This

[4] A culture of this organism was received under the name *Actinomyces bovis*. In the light of the current description of this species, however, it would appear that the organism is misnamed. The fact that it is entirely aerobic in its habit excludes it from the genus *Actinomyces* Harz, as defined at present (Waksman and Henrici, 1943). Furthermore, other morphological characters indicate its position in the genus *Streptomyces* Waksman and Henrici. Since the organism was utilized throughout the study, it was thought well to include the results obtained with it in this report under the designation used.

[5] Magneto-striction sonic oscillator (type R-221), Raytheon Manufacturing Co., used through courtesy of the Johnson Foundation, University of Pennsylvania.

fraction, removed from the centrifuge tube with a capillary pipette, served as antigen in the precipitin reactions. The use of the narrow centrifuge tubes facilitated the removal of the upper fraction from the tube because a relatively tall column of liquid was provided in such a tube as compared to that which would be found in a tube of considerably larger bore.

Animal inoculation. Several groups of mature rabbits were immunized with two types of antigen suspension, whole cells and sonically disintegrated cells. At least two animals were inoculated with each antigen, following the schedules outlined below.

Group I. Whole cells of several species of actinomycetes (tables 1 and 2), grown for 10 days in the nutrient broth medium, were repeatedly washed and centrifuged to remove all possible traces of the culture medium. The cells were killed with 5 per cent phenol in 0.85 per cent NaCl solution added to the washed cells. Animals were inoculated by the intravenous route, using the marginal ear vein, at weekly intervals with doses of antigen increasing from 0.1 ml to 0.5 ml, and then by 0.5-ml increments to a maximum of 2.0 ml. The 2.0-ml dose was continued at weekly intervals over a 2-month period, at which time the first bleeding from the heart was attempted. All animals were given 2.0-ml booster doses at 2-week intervals over a period of 5 months after the initial immunization period.

Group II. Suspensions of sonically disintegrated cells were used as sensitizing agents for a second group of animals. The same species of organisms were represented in this series as in group I, and the same schedule of inoculation was used as the one described for that group.

Group III. Several species of actinomycetes (tables 1 and 2) were grown in the synthetic medium of Long and Seibert (1926). This procedure was introduced in an attempt to avoid certain nonspecific precipitin reactions encountered in the initial attempts to demonstrate precipitins in the sera of animals treated with the antigens described above. Fuller discussion will be given in a later section of the paper.

Cells from 10-day cultures on the synthetic medium repeatedly washed with 0.85 per cent NaCl, and killed with 5 per cent phenol, were used as the immunizing agent for a third group of animals. Each animal received 2 inoculations on each of 7 consecutive days, 1.0 ml intravenously and 2.0 ml subcutaneously. At the end of the immunization period animals were rested for 14 days, after which samples of blood were removed from the heart.

Group IV. A fourth group of animals was immunized using antigen suspensions prepared from sonically disintegrated cells grown in the manner described for group III and following the same immunization schedule.

The reactive titer of sera from animals in groups III and IV, determined on the basis of the presence of agglutinins, was found to be either very low or entirely absent. All animals in both these groups were therefore given a second course of immunization following the same protocols described for those groups. At the completion of the second immunizing course agglutinin titers were demonstrated to have been increased considerably in every case.

TABLE 1

Agglutination reaction, showing highest reactive agglutinin titers of specific immune sera for all species of actinomycetes included in the study against the sonic antigen prepared from each species

SPECIFIC IMMUNE SERA	SONIC ANTIGENS											
	Nocardia asteroides	N. farcinica	N. madurae	Strep-tomyces aureus	S. flavovirens	S. griseus	S. lavendulae	S. reticuli	S. scabies	S. species	Actino-myces hominis	A. inter-medius
*Nocardia asteroides	1:5,120	1:40							1:160			
N. farcinica	1:80	1:320	1:40						1:40			
N. madurae			1:640						1:40			
Streptomyces aureus				1:640					1:20			
S. flavovirens					1:640		1:20		1:40			
*S. griseus						1:2,560	1:20	1:20	1:40			
S. lavendulae									1:40			
S. reticuli		1:20		1:40	1:40	1:40	1:40	1:1,280				1:80
*S. scabies	1:40								1:10,240	1:5,120		1:20
*S. species	1:40				1:20				1:80	1:20		1:20
Actinomyces hominis					1:20			1:20	1:80		1:1,280	
A. intermedius					1:20				1:40			1:640

* Antisera against these organisms prepared on long immunization schedule (groups I and II).

From 30 to 50 ml of blood were removed from each animal by cardiac puncture. The expressed serum was stored in sterile, rubber-stoppered vaccine bottles in the refrigerator until used. When the sera from several animals in a group that had been immunized with the same species of antigen showed comparable reactive titers, as tested by agglutinating power, the various samples of serum were pooled for future use. If, on the other hand, there was a wide difference in the agglutinating power between sera under the same circumstances, only the serum showing the highest titer was used in subsequent tests. It might be added that in only a few cases was there any significant difference in the reactive titer of sera from different animals that had been treated in a similar manner with corresponding antigenic material.

Technique of agglutination test. The macroscopic tube agglutination method was used throughout the course of the study. Immune sera were prepared in a series of dilutions in 0.85 per cent NaCl. The serum dilutions in the final test, after the addition of the antigen suspension, ranged from 1:20 to 1:1,280, the concentration in each succeeding tube differing from that in the preceding tube by one-half. The saline dilutions of the serum were made in 0.5-ml volumes. To each tube of diluted serum an equal volume of antigen suspension was added, the antigen suspension having been diluted with 0.85 per cent NaCl to correspond with the no. 1 tube of the MacFarland scale. The final volume in each tube of a test series was 1 ml. Racks holding the tubes were shaken to mix the ingredients thoroughly, incubated at 37 C for 2 hours, and refrigerated overnight. Readings of the results were made over a concave mirror, either in a direct beam of artificial light or in full sunlight. When positive agglutination could be noted in tubes containing serum diluted to 1:1,280, usually only in the homologous antigen and immune serum combination, a second test was set up carrying the serum dilutions to 1:10,240 in order to find the maximum agglutinating power of the serum (table 2).

The presence of a finely granular sediment at the bottom of the test tubes, which could be resuspended in the supernatant liquid with gentle agitation of the tube, and a simultaneous clearing of the uniform turbidity of the original antigen-serum mixture were considered to be a positive agglutination reaction if at the same time no such reaction was noted in the antigen-saline control tube.

Because of the frequent cross reactions observed in agglutinations, preliminary tests on the absorption of common agglutinins in the sera were attempted. Whole cells of those species of organisms that showed cross reactions with a particular immune serum were added to that serum and the suspension was incubated for 3 to 24 hours. After incubation of the suspension the cells were removed from the serum by centrifugation. When sera so treated were again tested for agglutinating power, however, it was noted that the reactive titer had been reduced considerably, not only for heterologous antigens but for the homologous antigen as well. The agglutination reactions with the absorbed sera thus gave results of no greater specificity but of less intensity than those with the unabsorbed sera. These data may indicate the presence in the crude antigen of a complex of antigenic materials. Prior to further studies on separation or

analysis of this crude antigen, attempts to absorb the serum either by whole cells or by complete sonic antigen may be considered of questionable value. Although the present undertaking has been directed chiefly toward the development of a serological method applicable to the actinomycetes, further studies on antibody absorption and analysis of the antigens are in progress.

Technique of precipitin test. Serial dilutions of antigens were prepared in 0.85 per cent NaCl in concentrations calculated to give final dilutions in the completed test ranging from 1:2 to 1:160. Each succeeding dilution, beginning with the second, which was 1:5, differed in antigen concentration from the one preceding by a factor of one-half. In those instances in which the positive reaction was noted in the highest antigen dilution used in the routine test, a second test was performed with additional higher dilutions of the antigen to find the highest reactive titer of a particular serum (table 3).

TABLE 2

Results of tests of the agglutinating power of various immune sera as measured against the homologous antigen

SONIC ANTIGEN	1:20	1:40	1:80	1:160	1 320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	CONTROL
*Nocardia asteroides..	++	++	++	++	+	+	+	±	±	—	—
N. madurae	++	++	+	+	+	+	—	—	—	—	—
Streptomyces aureus	++	+	+	+	+	+	—	—	—	—	—
S. flavovirens	++	++	+	+	+	+	—	—	—	—	—
*S. griseus	++	+	+	+	+	+	+	±	—	—	—
S. reticuli	++	++	++	+	+	+	±	—	—	—	—
*S. scabies	++	++	++	++	++	++	+	+	+	±	—
*S. species.	++	++	++	++	+	+	+	+	±	—	—
Actinomuces hominis	++	++	+	+	+	+	±	±	—	—	—

* Specific immune serum against these organisms was produced in animals treated on schedule outlined for groups I and II. Specific immune serum against species unmarked were produced in animals treated on the schedule outlined as groups III and IV.

In a modification of the method described by Brown (1938), the test mixtures were prepared on the inside surface of a thoroughly cleaned, scratch-free petri dish cover. A sufficient number of areas, approximately 1 centimeter square, were marked off with a glass-marking pencil on the inner surface of the cover to accommodate an average of five tests. The usual arrangement required a grid of 35 squares. To each square along the ordinate one drop of each of the antigen dilutions was added with a capillary pipette. To each square along the abscissa an equal quantity of immune serum was added in the same manner. The antigen and serum in each drop were mixed with a separate small capillary tube, the end of which had been closed in a Bunsen flame. After mixing, the petri dish cover was inverted over the lower section of the dish into which a disk of moistened filter paper had been placed. The test mixtures were incubated at 37 C for 2 hours, after which readings were made at a magnification

of 100 ✕. For reading, the petri dish lid alone was placed on the stage of the microscope. After the preliminary reading the plates were allowed to remain at room temperature overnight, after which time a second reading was made.

The appearance of finely granular, opaque material evenly distributed through the test drop as viewed through the microscope was taken to represent the formation of a precipitate if in the serum and antigen controls no such material could be observed after treatment similar to that given the test mixtures.

The results of the agglutination and the precipitin tests as described above are given in tables 1, 2, and 3. Table 1 shows the results of agglutination reactions observed between the antiserum prepared against each of the species of actinomycetes included in the study, with antigens prepared from all the species.

TABLE 3

Precipitation reaction, showing highest reactive precipitin titers of specific immune sera for several species of actinomycetes against sonically prepared antigens

SPECIFIC IMMUNE SERA	SONIC ANTIGENS							
	Nocardia asteroides	*N. farcinica*	*N. madurae*	*Streptomyces flavovirens*	*S. reticuli*	*S. scabies*	*·S.* species	*Actinomyces hominis*
Nocardia asteroides.	1:40					1:2		
N. farcinica.........	1:2	1:40						
N. madurae........			1:20					
Streptomyces flavovirens ...				1:20		1:5		
S. reticuli.............. ...					1:2			
S. scabies............. ..						1:640	1:5	
S. species........						1:10	1:1,280	
*Actinomyces bovis . .								
A. hominis....								1:20

* Serum received from Dr. John M. Slack.

Table 3 shows the results of the precipitin reactions between each of several antigen preparations and several specific antisera. Only highest reactive titers for both agglutinating and precipitating sera are given in tables 1 and 3. The intensity of the positive reactions in table 2 is indicated by the use of a plus sign, double plus denoting a very strong reaction, single plus a moderately strong reaction, plus and minus a weak positive reaction, and minus the absence of an observable reaction.

DISCUSSION

In any serological approach to the actinomycetes, the problem of the preparation of satisfactory antigens, particularly those to be used in agglutination studies, becomes immediately apparent. The problem arises out of the fact that the organisms involved are often very long, branched structures, the individual cells of which are not readily separable. Even when the organisms are not especially filamentous, the cells of all species studied are uniformly resistant to disruption. These characteristics prevent the preparation, by ordinary methods,

of cell suspensions satisfactory for use as antigens. Cultures grown in liquid media produce masses of intertwined filaments, the components of which cannot readily be freed one from the other. Even when extremely young cultures are employed, before the cells have attained any considerable length, tangling of the short filaments results as soon as the cells settle out of suspension. When cultures are grown on solid media, the masses of growth are entirely resistant to separation into suspensions of free cells by ordinary mechanical means. The individual filaments, during the development of colonies, become so intimately intergrown, and the resulting structure is usually so tough and sometimes almost sclerotiumlike, that a separation of the cells is impractical.

Although a suspension of whole cells may be entirely satisfactory for animal immunization, their marked tendency to aggregate precludes their use as antigens with the immune serum in an agglutination test.

In the early phases of this work the methods for the preparation of antigen suspensions described by other workers were followed. Cells harvested from liquid or solid media were ground in a mortar with sand, and others were subjected to ball-mill grinding. Such treatments failed to produce a satisfactory volume of finely divided cell material even after a reasonably extended period of grinding.

In view of the failure of these methods properly to break up the cells, disintegration by sonic vibrations was chosen as the method to produce a satisfactory antigen. As described above, the sonic apparatus produced a completely homogeneous suspension of fragmented cell material that could be handled in the same manner as a suspension of smaller, more uniform cells. From this suspension an eminently satisfactory antigen could be prepared for both the agglutination tests and the precipitin tests.

It will be immediately apparent that the suspension of cellular material produced by sonic vibrations differs considerably from a suspension of whole cells. In the suspension taken from the sonic apparatus, in addition to the small fragments of the cell envelope itself, there is also present all of the cytoplasmic material of the cell. Presumably the water-soluble fractions of the cell, as well as the insoluble cell fragments, will be reactive with specific serum antibodies. Indeed this is demonstrated in the fact that in the clear supernatant fluid obtained from centrifuging the sonic product are substances that will react with serum precipitins to form an observable precipitate. That these substances do not interfere with a clear-cut agglutination reaction, however, was amply demonstrated by the numerous successful agglutination reactions performed during the course of the investigation. It is not inconceivable that whatever precipitates may be produced are so intimately intermixed with the agglutinated cell fragments in the agglutination tubes that they appear as part of the granular complex seen in the positive reaction. More significant perhaps is the fact that, in those series in which the agglutination reaction was read as negative, nothing in the nature of a precipitate could be observed by the method of reading the test which would cause confusion in the interpretation of the result. Noteworthy in this connection is the work reported by Jones (1927) and Arkwright

(1914), among others, in which the agglutinating properties of precipitins are demonstrated in systems in which it is possible for the precipitate-producing antigen to be adsorbed on the surface of some particulate material. It might be considered that a similar process was operating in the system discussed above.

It is the opinion of the authors that the agglutination and precipitation techniques, as described here, present themselves as readily applicable methods for the serological study of the actinomycetes. The use of sonically prepared antigens has removed what may reasonably be considered to have been an obstacle in former attempts to study the actinomycetes by these particular serological techniques. These methods may be recommended on the basis of the comparative ease of preparation and manipulation of the materials involved and the ease with which the results may be read by one experienced in the use of serological techniques.

The nature of the material used as the antigen for the immunization of test animals, either killed whole cells or suspensions of these cells disintegrated by sonic vibration as employed in the present work, seems to have no appreciable influence on the antibody titers attained in the animals. Sera of comparable reactive titer were produced in groups of animals immunized with either type of preparation. It is worthy of note, however, that the antigen consisting of the disintegrated cells was much more readily used than the suspensions of whole killed cells, through the avoidance of mechanical difficulties commonly encountered while injecting the latter preparation into animals.

Of special importance in the precipitin test is the nature of the culture medium used in growing the cells from which the antigen suspensions are to be prepared. Culture media containing antigenic ingredients must be avoided since these may be introduced into the animal along with the cellular antigen. This is especially essential when the same antigen preparation is to be used in testing for precipitating antibodies in serum. This fact has been strikingly demonstrated by Slack (1948), working with several strains of *Actinomyces bovis*. He has shown that cells grown in Brewer's anaerobic medium, even after repeated careful washing, carry with them, when used in rabbits, enough of the antigenic materials from the medium to elicit the formation of precipitins in the animals. This demonstration makes it obvious that if the substances from liquid culture media may remain in association with cellular material, even through a careful washing process, methods employing solid culture media for the production of cellular antigens (Claypole, 1913) are to be rigorously avoided. This is particularly essential in the case of actinomycetes since the separation of these colonies from the solid substrate is extremely difficult if not impossible. In the present work, either the cells that were used for animal immunization or the cells from which the antigens for the various tests were prepared, were grown in antigen-free synthetic broth medium. When possible, cells to be used for both types of antigens were cultivated in synthetic broth media.

Of equal importance with the choice of the method of preparation for immunizing materials is the schedule of animal immunization. It seems to have been the experience among the earlier investigators already cited, as well as in

the present study, that the successful production of high-titer immune serum depends upon a relatively long period of immunization. As indicated in the tables of results, those animals that were immunized on long-period schedules (groups I and II) produced immune sera of higher reactive titer generally than those whose schedules of immunization were more rapidly concluded. This would appear to be true even though the same preparations and approximately the same total dosages were used in each case.

The results of the agglutination tests may be interpreted as indicating the presence of at least two antigen complexes in the suspensions used. The first of these may be considered to bring about cross reactions between a particular specific immune serum and heterologous antigen suspensions of organisms within a genus. The extent to which such cross reactions may indicate interspecies relationship and be used to confirm existing taxonomic systems cannot be indicated at present. That there may be also some relationship between individuals from different genera may be suspected from the cross reactions that occur between a particular specific immune serum and the antigen suspension of a species of another genus, i.e., *Streptomyces scabies* and *Nocardia asteroides* (table 1).

The second of the two antigen complexes, which may be thought of as the specific antigen, may be considered as indicated in the high-titer homologous agglutination reactions, data from which are recorded in tables 1 and 2. When agglutinating serum of high reactive titer was used, it was always possible to separate the homologous antigen from among several heterologous antigens on the basis of the relatively high dilution of the specific immune serum in which positive agglutination could be observed. Among the organisms used in this study these two factors always served adequately to distinguish the homologous antigen from related antigens when several antigens were tested against a known specific antiserum. This reaction should prove useful, therefore, as an aid in the identification of cultures of actinomycetes and in determining the group relationships of such cultures when these facts are in doubt.

With respect to the relatively low activity of precipitins in the several immune sera tested, at least two possible explanations may be offered (table 3). The first of these is that already considered above in which the lack of high reactive titer in the serum is attributed to the rapid, short-term immunization schedule followed in producing the sera. As has been stated, it seems necessary to subject the animals to relatively long periods of immunization with actinomycetes if high-titer immune sera are to be produced. As indicated, the sera used in the precipitin reactions were taken from rabbits immunized on the rapid schedule described above (groups III and IV).

Another factor that may be considered in this respect is the method chosen to prepare the antigens for these tests. Only those cell substances that are water-soluble would be present in the antigen. In the work of others who have reported precipitin reactions of high titer, the antigenic material had been prepared in a manner that would provide for the presence in the antigen of substances other than those that are only water-soluble. Goyal (1938), for instance,

has used ethyl alcohol extracts of the cells as precipitin antigens, and Claypole (1913) used absolute alcohol to extract dried cells in the preparation of a precipitate-forming antigen. The comparison of results obtained when antigens prepared by several methods were used might prove to be of value in connection with this question.

SUMMARY

Sonic vibrations may be used to produce an antigen suspension from the cells of actinomycetes satisfactory for use in agglutination and precipitin studies on members of this group of microorganisms.

The antigen suspensions produced by sonic vibrations may be used successfully, following appropriate inoculation schedules, for the production in mature rabbits of specific immune sera against the actinomycetes.

It is probable that the techniques of agglutinin and precipitin reactions may be developed to serve as aids in the identification of, and as a means for demonstrating relationships within, the actinomycetes.

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GROWTH INHIBITION OF EBERTHELLA TYPHOSA BY CERTAIN CARBOHYDRATES AND ITS RELEASE BY MUTATION[1]

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The present article concerns an investigation of a case of bacterial variation described as early as 1911 by R. Müller, who observed that colonies of *Eberthella typhosa* on nutrient agar containing L-rhamnose—a methyl pentose—gave rise to secondary colonies or "papillae" after 4 or 5 days of incubation at 36 C. According to Müller, this papillation was one of the most constant characteristics of typhoid bacilli. Neither the normal strains nor the mutants isolated from papillae produced acid or gas from L-rhamnose. The sugar inhibited growth of the normal strains but not of the mutants isolated from the papillae. Müller suggested that either the mutants utilized rhamnose without acid production, or rhamnose inhibited growth of the normal strains and not of the mutants. Of 19 other carbohydrates tested, only L-arabinose caused papillation of "typhoid-like" bacilli, not of *E. typhosa* itself.

Stephenson (1939) assumed that the rhamnose mutation involved ability to utilize the sugar and compared it with the lactose mutation in *Escherichia coli-mutabile*. Kristensen (1944) demonstrated that single cell cultures of various strains of *E. typhosa* gave rise to secondary colonies in the presence of L-rhamnose. Lwoff (1946) listed the rhamnose mutation as an example of "anaphragmic mutation," a mutation involving the "suppression of an inhibition." Lwoff assumed that the normal *E. typhosa* attacks L-rhamnose and is inhibited by the sugar or by some of its breakdown products, whereas the mutant fails to attack the sugar.

The work described in the present paper shows that the normal and the rhamnose mutant strains of *E. typhosa*, both unable to utilize rhamnose, differ only in susceptibility to inhibition by the sugar. The rhamnose mutation has been found to be one of a number of mutations affecting response to a group of carbohydrates, which owe their specific activity to their L configuration.

MATERIALS AND METHODS

Cultures. *E. typhosa* strain IMS was received from the School of Medicine, Indiana University, Indianapolis. *E. typhosa* 0–901 (Vi negative) was kindly supplied by Dr. J. M. Cragie.

Media. Difco nutrient broth and nutrient agar were used for routine cultivation. The following mineral base was employed to investigate the ability of

E. typhosa IMS and of its mutant strains to utilize various sugars as sources of carbon: MgSO$_4$·7H$_2$O, 0.2 g; NH$_4$Cl, 1 g; M/15 phosphate buffer pH 7.4, 1,000 ml. This solution was sterilized by autoclaving. L-Tryptophan, sterilized separately by filtration, was added to give a final concentration of 25 micrograms per ml. Sterile carbohydrate solution was added to give a final concentration of 0.2 per cent. Since growth in this medium was slow, a supplement of acid hydrolyzate of casein (SMACO) was added in several experiments to give a final concentration of 0.01 per cent. Carbohydrates were sterilized in aqueous solution for 10 minutes in the autoclave, unless otherwise specified, and added aseptically to the media. All cultures were incubated at 37 C.

RESULTS

The first experiments confirmed Müller's observations. *E. typhosa* IMS streaked on nutrient agar containing 1 per cent L-rhamnose gave smaller and

TABLE 1

Viable counts of E. typhosa, strains IMS and IMS/R, in nutrient broth with or without L-rhamnose or L-arabinose

(Ten-ml cultures in 125-ml flasks shaken at 37 C. Values in viable cells per ml, from colony counts in nutrient agar)

	STRAIN					
	IMS			IMS/R		
TIME	No sugar	L-Rhamnose 1%	L-Arabinose 1%	No sugar	L-Rhamnose 1%	L-Arabinose 1%
hours						
0	1.6 × 10^4	1.6 × 10^4	1.6 × 10^4	1.4 × 10^4	1.4 × 10^4	1.4 × 10^4
11	8.0 × 10^8	2.4 × 10^8	3.8 × 10^8	3.0 × 10^8	7.0 × 10^8	7.0 × 10^7
17	2.4 × 10^9	2.5 × 10^8	7.7 × 10^8	1.8 × 10^9	1.0 × 10^9	3.5 × 10^8
23	3.2 × 10^9	5.8 × 10^8	1.5 × 10^9	3.0 × 10^9	2.4 × 10^9	5.9 × 10^8
40	—	—	—	2.4 × 10^9	1.6 × 10^9	3.8 × 10^8

thinner colonies than on control plates without sugar. After 2 days all colonies on rhamnose agar showed numerous yellowish, opaque papillae, which 1 day later had overgrown the original colonies. Papillae were formed in 48 hours with L-rhamnose in concentrations as low as 0.1 per cent. The mutant type IMS/R was isolated by touching the edge of a papilla with a sterile needle and streaking on rhamnose agar, on which the mutant gave nonpapillated colonies.

In nutrient broth, growth inhibition of strain IMS was evident by visual observation at a concentration of 0.2 per cent rhamnose and maximal at concentrations of 0.6 per cent or higher. Strain IMS/R is not inhibited by L-rhamnose in concentrations as high as 5 per cent. These results were confirmed quantitatively by the experiments exemplified in table 1, which show the somewhat reduced viable titers reached by cultures of strain IMS in rhamnose broth (and in arabinose broth, see below).

Effect of various sugars on growth of E. typhosa. A number of sugars (D-glucose, D-lactose, D-sucrose, L-arabinose, D-ribose, D-xylose, L-fucose, L-sorbose, and D-

mannose) were incorporated singly in nutrient agar and tested for the ability to cause papillation in strain IMS. On lactose, sucrose, ribose, xylose, and mannose strain IMS gave colonies identical to those on plain nutrient agar. Glucose in concentrations above 0.2 per cent gave smaller colonies, possibly because of the accumulation of acid degradation products.

In the presence of L-arabinose, papillae similar to those formed on L-rhamnose appeared within 48 hours. Arabinose mutants from the normal strain (IMS/A) and from the rhamnose mutant (IMS/R/A) were obtained in pure culture from papillae. Strains IMS/A and IMS/R/A are not inhibited and do not form papillae on arabinose agar.

On fucose agar the colonies appeared to be inhibited, but no papillae were observed. On sorbose agar the colonies were smaller and showed some papillae after 5 days. A strain IMS/S isolated from a papilla grew well on sorbose agar without forming papillae.

Strains IMS gave papillated colonies also on agar to which rhamnose (or arabinose), sterilized by filtration rather than by autoclaving, had been added. Rhamnose and arabinose mutants were also isolated from *E. typhosa* strain 0-901.

Relation between rhamnose and arabinose mutations. The fact that strain IMS/R gave rise to a mutant IMS/R/A detectable on arabinose agar indicated that two different mutations are involved. The arabinose mutant IMS/A gave rise on rhamnose to mutant colonies IMS/A/R. Separate mutations are evidently responsible for the two changes. The double mutants do not show papillae on agar containing either L-rhamnose or L-arabinose.

Ability to utilize different sugars as carbon sources. The ability of strains IMS and of its mutants to grow in a synthetic medium with various carbohydrates as the sole source of carbon was tested, the same basal medium with glucose as a substrate serving as a control. Inocula of various sizes were used, containing from 10^3 to 10^7 organisms. The results demonstrated that none of the strains can utilize either L-rhamnose, L-arabinose, or L-sorbose as the sole carbon source. Strain IMS is unable to utilize L-fucose.

Acid production from various sugars. Acid production by strain IMS and its mutants growing in the presence of various sugars was tested using cultures in nutrient broth containing 1 per cent sugar. The results, shown in table 2, indicate that neither the parent strain nor the mutants produce acid from rhamnose, arabinose, or sorbose, whereas all do so from glucose.

Oxidation of various sugars. Since carbohydrates may be oxidized by microorganisms without acid production, the action of the parent strain and of the mutants on rhamnose, arabinose, and sorbose was tested manometrically. The organisms were grown for 16 to 18 hours on either plain nutrient agar or nutrient agar containing 0.3 per cent of a specific sugar, after which the cells were harvested, washed by centrifugation, and resuspended in M/15 phosphate buffer at pH 7.0. All experiments were conducted at 37 C in an atmosphere of air, using the Warburg apparatus. Each vessel contained 2.0 ml of cell suspension in the main compartment and 0.2 ml KOH in the center well; oxygen consumption

was measured after the addition of 5 micromoles of sugar from the side arm. In each set of experiments a test with glucose was included to provide a control on the activity of the cell suspension. Figure 1 shows that none of the strains tested utilized either L-rhamnose, L-arabinose, or L-sorbose.

Tests for the effect of L-rhamnose and L-arabinose on various metabolic processes. The possible interference with glucose oxidation was first tested. Figure 1A,B shows that rhamnose has no effect on the oxidation of glucose by either strain IMS or strain IMS/R; likewise, arabinose does not affect glucose oxidation by either strain IMS or IMS/A (figure 1C,D). These sugars do not affect ribose oxidation (figure 1F).

TABLE 2

Production of acid in cultures of E. typhosa strain IMS and of its mutants

(Cultures in 5 ml of nutrient broth containing 1 per cent carbohydrate were incubated at 37 C for 6 days and titrated to neutrality with 0.1044 N KOH using phenolphthalein as indicator)

CARBOHYDRATE	NONINOCU- LATED CONTROL	IMS	IMS/R	IMS/A	IMS/S
	ml KOH needed to neutralize 5 ml of culture				
None	0.25	0.20	0.10	0.10	0.15
L-Rhamnose	0.25	0.10	0.15		
L-Arabinose	0.25	0.10		0.10	
L-Sorbose	0.25	0.10			0.05
L-Fucose	0.25	0.10			
D-Ribose	0.30	0.70			
D-Glucose	0.20	0.75	0.80	0.85	0.85

Since rhamnose clearly inhibits the growth of the normal strain in nutrient broth, its effect on the oxidation of peptone was tested. The results, illustrated in figure 2A,B show that rhamnose does not interfere with the oxidation of peptone by either the normal or the rhamnose mutant. The mechanism of the inhibitory action of the sugars remains, therefore, an open question.[3]

Role of the steric configuration of the sugars. The sugars which proved inhibitory and led to the isolation of mutants all belonged to the L-series of steric configuration. Secondary colonies were not observed in the presence of any sugar of D configuration, but colonies on L-rhamnose, L-arabinose, and L-sorbose agar gave rise to papillae. L-Fucose inhibits growth but does not cause papillation, and it is conceivable that no specific mutation capable of overcoming fucose inhibition occurs. To verify the role of the steric configuration of the

[3] In experiments with strain IMS streaked on agar containing both rhamnose and glucose, an interesting effect was observed: 1 per cent glucose suppressed formation of papillae and 0.2 per cent glucose delayed it, but 0.04 per cent glucose had no effect. This interaction is probably due to the production of acid from glucose; the colonies of IMS (and of IMS/R) on glucose agar are smaller and denser than those on nutrient agar, and it is possible that local concentration of acid products inhibits growth and prevents the late appearance of secondary colonies.
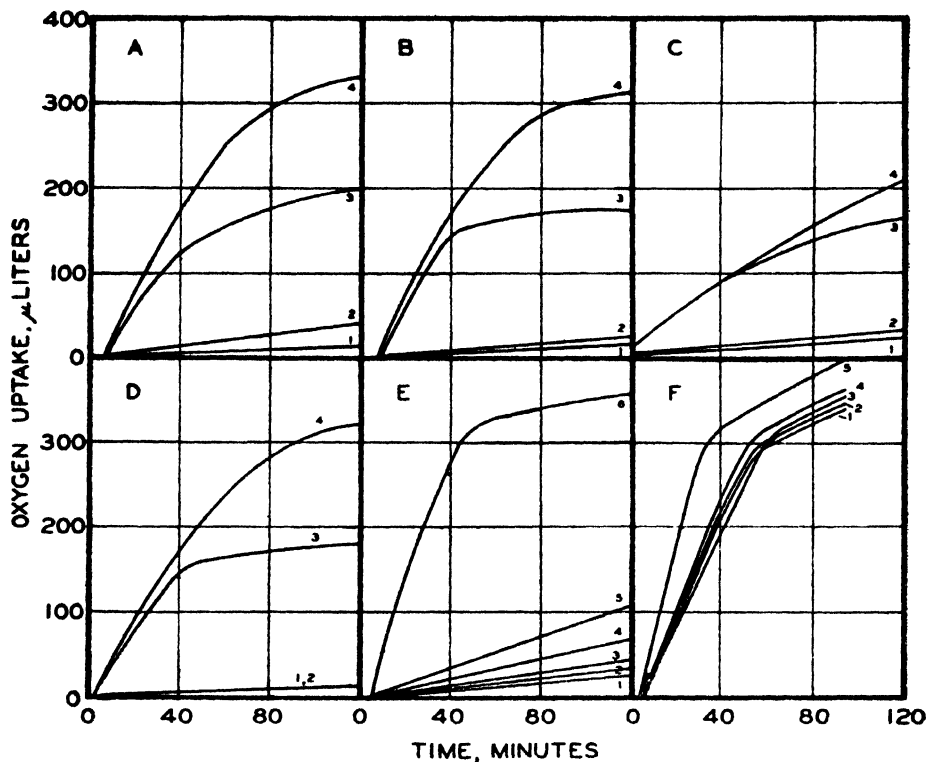
Figure 1. *Diagram A*. Oxygen uptake by strain IMS grown on L-rhamnose agar: (1) 5 micromoles of L-rhamnose; (2) autorespiration; (3) 2.5 micromoles of glucose plus 2.5 micromoles of L-rhamnose; (4) 5 micromoles of glucose. *Diagram B*. Same as A, but for strain IMS/R. *Diagram C*. Same as A, but for strain IMS grown on L-arabinose agar, using L-arabinose instead of L-rhamnose in the tests. (Substrate utilization was incomplete in 120 minutes, because a light bacterial suspension was used.) *Diagram D*. Same as C, but for strain IMS/A. *Diagram E*. Oxygen uptake by strain IMS grown on nutrient agar: (1) autorespiration; (2) 5 micromoles of D-rhamnose; (3) 5 micromoles of D-arabinose; (4) 5 micromoles of L-sorbose; (5) 5 micromoles of D-ribose; (6) 5 micromoles of D-glucose. *Diagram F*. Oxygen uptake by strain IMS grown on D-ribose agar: (1) 5 micromoles of D-ribose; (2) 5 micromoles of D-ribose plus 5 micromoles of L-rhamnose; (3) 5 micromoles of D-ribose plus 10 micromoles of L-rhamnose; (4) 5 micromoles of D-ribose plus 5 micromoles of L-arabinose; (5) 5 micromoles of D-glucose.
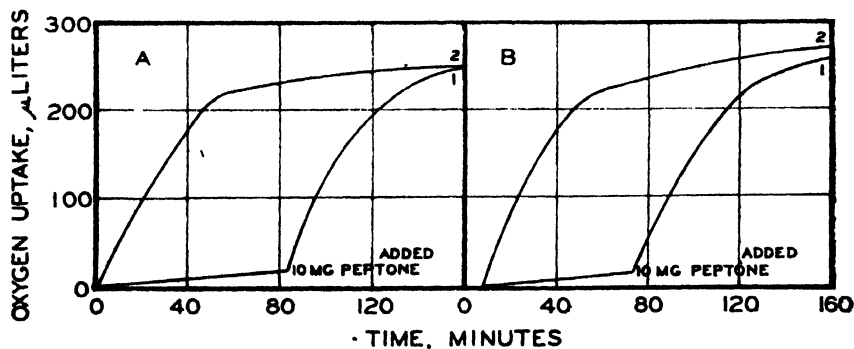
Figure 2. *Diagram A*. Oxygen uptake by strain IMS grown on nutrient agar. *Diagram B*. Same for strain IMS/R: (1) 10 mg of L-rhamnose plus 10 mg of peptone, the latter added 80 minutes after the beginning of the experiment; (2) 10 mg of peptone, either with or without 10 mg of L-rhamnose added simultaneously.

sugars, we tested both D-rhamnose and D-arabinose.[4] Colonies of strain IMS on agar containing these sugars show neither inhibition nor papillation.

Most of the naturally occurring sugars possess the D configuration and examples are known of microorganisms utilizing the D form but not the L form of a sugar. Tests with D-rhamnose and D-arabinose showed, however, that D-rhamnose and D-arabinose were neither utilized as a sole carbon source nor attacked with the production of acid. Manometric determinations (figure 1E) showed that strain IMS is unable to oxidize these sugars.

DISCUSSION

The experiments indicate that the IMS strain of E. typhosa shows growth inhibition by at least four carbohydrates of L configuration. Inhibition by each of three sugars can be relieved by specific mutations; the papillae formed on agar containing the sugar stem from noninhibited mutants. The inhibitory L-sugars are apparently not attacked either by the normal strain or by its mutants, nor is any attack on the noninhibitory D-isomers detectable, at least in the case of rhamnose and arabinose. We realize that complete proof of non-utilization could only have been obtained by demonstration of the presence of the carbohydrates in unchanged amounts in culture media after growth, but such tests were unfortunately not performed. Another desirable test would have been that of the L-isomers of some utilizable carbohydrates, but we were unable to secure any of them.

Our experiments have failed to reveal the mechanism by which the L-sugars cause growth inhibition of E. typhosa. The inhibition, never very pronounced and mostly evident in late phases of growth, might concern some synthetic reaction, possibly the synthesis of an essential carbohydrate, as suggested by Lwoff (1946). If an enzymatic block is involved, reduced growth may result from the utilization of a less efficient bypath.

The remarkable fact is that the mutations releasing inhibition by different L-sugars are specific for each sugar. Each of the L-sugars appears to cause a specific block by affecting either a different enzyme or a different portion of a certain enzyme. If the inhibition were exerted by all L-sugars on the same reaction and if the various mutations releasing the inhibition produced alternative configurations of the same enzyme, the production of double mutants, e.g., IMS/R/A, would be difficult to explain. The mutant isolated from IMS/R on L-arabinose should be phenotypically IMS/A rather than IMS/R/A.

Mutation rates were not determined in our study because of the difficulty in detecting, by plating methods, mutants present in the original population of the normal strain. By the time that normal and mutant colonies on agar containing the inhibitory sugar are distinguishable, the papillae appear and prevent the counting of colonies originated from mutant cells. Judging from the frequency of the papillae, the proportion of mutated cells is probably rather low.

The mutations can be defined as "anaphragmic" in the sense employed by

[4] The D-rhamnose and the D-arabinose were generously supplied by the laboratory of Dr. C. S. Hudson, National Institute of Health, Bethesda, Maryland.

Lwoff (1946) provided we remember that the term is merely descriptive of the over-all result of the mutation without implications as to mechanism, since actual inhibition—and release of inhibition—of specific metabolic steps has not been proved.

SUMMARY

Growth of *Eberthella typhosa* (strain IMS) is somewhat inhibited in media containing any one of four L-carbohydrates that were tested, namely, L-rhamnose, L-arabinose, L-sorbose, and L-fucose. No inhibition is evident in the presence of D-carbohydrates, including D-rhamnose and D-arabinose. On nutrient agar containing L-rhamnose, L-arabinose, or L-sorbose the slightly inhibited colonies form papillae, from which noninhibited mutants can be isolated. Each mutation releases the inhibition by one L-carbohydrate only. The inhibitory L-carbohydrates and their D-isomers are not attacked by the normal strain or by any of its mutants. Each carbohydrate appears to act as a specific inhibitor, but the mechanism of inhibition has not been identified.

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NOTES

TWO NEW SALMONELLA TYPES: SALMONELLA CORVALLIS AND SALMONELLA COLORADO

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Salmonella corvallis was isolated by Dr. E. M. Dickinson from the pooled cecal contents of poults affected with enteritis. The culture was forwarded to the writers by Dr. W. R. Hinshaw. Upon examination the organism was found to be a motile bacterium that possessed the usual cultural, tinctorial, and biochemical attributes of the genus *Salmonella*. Glucose, arabinose, xylose, rhamnose, maltose, trehalose, inositol, mannitol, and sorbitol were fermented promptly with the production of acid and gas. Cellobiose and dulcitol were fermented after 6 days' incubation. Lactose, sucrose, salicin, and adonitol were not attacked. Citrate and D-tartrate were utilized and hydrogen sulfide was formed, but indole was not produced nor was gelatin liquefied.

Serological examination revealed that the O antigens of *S. corvallis* were closely related to those of *Salmonella kentucky* (VIII, XX), but in absorption tests *S. corvallis* left a slight residue of agglutinins for *S. kentucky* and *Salmonella newport* (VI, VIII) in *S. kentucky* O serum. When *S. newport* O serum was absorbed by *S. corvallis* a strong titer for *S. newport* and a weak titer for *S. kentucky* remained. Although the organism lacks a small fraction of antigen VIII, the O antigens of *S. corvallis* are well represented by the symbols VIII, XX.

The H antigens of *S. corvallis* were monophasic and were identical with those of *Salmonella cerro* (z_4, z_{23}). The antigenic formula of *S. corvallis* is VIII, XX: z_4 z_{23}.

Salmonella colorado was isolated from a stool specimen in the laboratories of the Colorado State Department of Health and was forwarded to this laboratory by Miss Marjorie Van Vranken. The clinical condition of the person from whose stool it was isolated is not known.

The biochemical properties of *S. colorado* differed from those given for *S. corvallis* only in that dulcitol was fermented promptly and inositol was not attacked. Serologically it was a member of group C of the Kauffmann-White classification, and in absorption tests it removed all agglutinins from *Salmonella thompson* (VI, VII) O serum.

The H antigens of *S. colorado* were diphasic, and phase 1 was found to be identical with phase 1 of *Salmonella worthington* (l,w). Phase 2 was agglutinated by serums derived from the nonspecific phases of the genus. When tested with serums for single factors, 2, 5, 6, and 7, it was agglutinated only by serum for factor 5. In absorption tests *S. colorado* reduced the titer of serum for phase 2 of *S. thompson* from 20,000 to 200. Phase 2 of *S. colorado* may be represented

by the symbols 1, 5, and its antigenic formula is VI,VII:1,w-1,5. This is the second *Salmonella* type in which antigens 1,w were found in combination with a nonspecific phase; the first is *Salmonella fayed* of Anderson, Anderson, and Taylor (J. Path. Bact., **59,** 533, 1947).

ADDITIONAL PROPERTIES OF THE MEF1 STRAIN OF POLIOMYELITIS VIRUS, ESPECIALLY WITH REFERENCE TO ATTEMPTS AT CULTIVATION IN THE CHICK EMBRYO

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Successful cultivation in fertile hens' eggs has been reported of Theiler's virus, TO, FA, GDVII strains, but not of poliomyelitis virus, Lansing, Y-SK, Ph, and several other human strains (Riordan and Sa-Fleitas: J. Immunol., **56,** 263, 1947; Dunham and Parker: J. Bact., **45,** 80, 1943; and others). These findings on TO, FA, and GDVII and on Lansing viruses have been confirmed in this laboratory.

In view of the fact that multiplication in chick embryos is a distinct feature separating so-called murine from poliomyelitis viruses and since the MEF1 strain (Schlesinger, Morgan, and Olitsky: Science, **98,** 452, 1943) has not as yet been studied in this regard, the present report on such studies including certain other properties is presented.

Chick embryos, 7 to 11 days old, were inoculated with a 20 per cent MEF1 virus as a mouse brain suspension, 0.03 ml intracerebrally, 0.25 ml into the yolk sac, or 0.1 ml on the chorioallantoic membrane (C.A.). The membranes were then incubated for 7 to 11 days, the yolk sacs for 11 days, and the intracerebral series, 7 days. The C.A. or embryo (or both) and brain in 10^{-4} dilution were subinoculated intracerebrally in mice. Blind passage from the inoculated embryos to 2 or more series of normal chick embryos was made along with subinoculation in mice. The result was that MEF1 virus was found to be incapable of multiplying in chick embryos even intracerebrally. It is of interest that in one instance in the C.A.-inoculated series the membrane in 10^{-1} dilution induced paralyses in the mice. Although the mouse brain virus was identified by positive neutralization with Lansing antiserum, it was proved by passage that the virus did not multiply in the membrane but that it only persisted in the inoculum.

In addition, the MEF1 after a large number of mouse passages exhibits a higher LD_{50} titer after intracerebral inoculation in the Rockefeller Institute strain of mice, viz., 3.0 to 4.2, than it does in the Lansing strain, which after many more mouse passages still shows the LD_{50} titer not to exceed 3 and at times

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less than 2.   An added factor is the uniformity with which these animals respond to low dilutions of MEF1 virus as compared with the irregularity of reactors to those of the Lansing strain.   This offers an advantage for certain experiments with rodent-adapted poliomyelitis viruses.

VITAMIN REQUIREMENTS OF BACILLUS COAGULANS

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During studies on some thermophilic members of the genus *Bacillus*, it was found that *Bacillus coagulans* NRS 27, a eurithermophile, could be serially cultivated at 37 C and at 55 C in a medium of the following composition: vitamin- and salt-free casein hydrolyzate, 1 per cent; L-cystine, 0.001 per cent; DL-tryptophan, 0.01 per cent; NaCl, 0.1 per cent; D-glucose, 0.5 per cent; K_2HPO_4, 0.5 per cent; thiamine, 1 μg per ml; niacin, 1 μg per ml; biotin, 0.04 μg per ml.

TABLE 1

Growth of B. coagulans NRS 27, at 37 C and 55 C, in casein hydrolyzate medium, with combinations of niacin, thiamine, and biotin

VITAMIN CONCENTRATION, µg/ML			GROWTH RESPONSE	
Niacin	Thiamine	Biotin	37 C	55 C
1	1	0.04	66*	53*
1	1	0.004	62	46
1	1	0.00004	41	25
1	1	0.0000004	10	0
1	1	0	0	0
1	0.01	0.04	0	0
1	0	0.04	0	0
0.1	1	0.04	66	53
0.01	1	0.04	62	51
0	1	0.04	0	0
0	0	0	0	0

* Average of replicate serial 48-hour transfers.   Figures represent turbidity as measured with Fisher electrophotometer, 100 minus reading of light transmittance, using 425 B filter.

Table 1 shows the growth response of this organism to the three vitamins in the medium described above.

The results indicate that this organism requires a relatively high concentration of the three vitamins reported and each of these vitamins is essential

for growth. Growth in the casein hydrolyzate medium with niacin, thiamine, and biotin is less prompt and less abundant than in trypticase soy broth; but the spore yield, which is consistently low for this organism, is equal in both media. At the lower temperature, more spores are formed; the cells are wider and shorter, and stain more evenly with Giemsa stain.

FACTORS AFFECTING THE ELABORATION OF PIGMENT AND POLYSACCHARIDE BY SERRATIA MARCESCENS

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The purpose of this note is to confirm and extend the interesting observations recently reported by J. P. Duguid (J. Path. Bact., **60**, 265, 1948). He has demonstrated very clearly that capsule formation in *Aerobacter aerogenes* is encouraged whenever growth is checked by limiting quantities of a nitrogen source or phosphate, provided an excess of carbohydrate is available. Under such conditions capsules are large and the cells, depleted of their cytoplasmic ribonucleotide, reveal their nuclei very conspicuously when stained with basic dyes.

We have made essentially the same observations with *Serratia marcescens*, using the Hy strain, which has typical coccoid cells. The results were particularly clear when the cultures were abundantly aerated on a shaker. In a simple medium with 0.5 per cent peptone, 1.0 per cent glycerol, and 0.1 per cent K_2HPO_4, or Na_2HPO_4, there was little evidence of capsule formation, the cells stained uniformly with basic dyes, and pigmentation was scarcely evident. However, when the peptone was reduced to 0.1 per cent, or when the added phosphate was omitted from the medium, the results after 48 hours at 30 C were very different. A great deal of capsular material was elaborated; simple basic dyes such as methylene blue revealed deeply staining nuclear bodies in cytoplasm that stained very faintly indeed; pigmentation was intense. The identity of the nuclei was established with Giemsa and Feulgen preparations.

Moreover, when smears of cells grown for 2 or more days on the shaker in peptone glycerol medium lacking added phosphate were treated with periodic acid and exposed to Schiff's reagent (McManus: Nature, **158**, 202, 1946), the cytoplasmic material surrounding the nuclei was stained an intense pink. The nuclei appeared as unstained zones in the cells. The cells were not stained by Schiff's reagent when the periodate treatment was omitted. This recolorization of Schiff's reagent following treatment with periodic acid has been ascribed to the presence of polysaccharide material (Hotchkiss: Arch. Biochem., **16**, 131, 1948). Young cells from the same cultures did not give a positive periodate-Schiff's reaction; these cells were not highly pigmented and gave little evidence of

capsules. Similarly, cells grown in peptone glycerol broth with added phosphate did not contain appreciable amounts of stainable polysaccharide material. Growth was good and pigment and polysaccharide were formed readily when mannose, galactose, sucrose, or maltose was substituted for the glycerol; but when glucose was used, growth and pigmentation were poor and cellular morphology atypical, unless calcium carbonate or another buffer was present to control the high acidity that developed.

Aerobacter aerogenes was also grown on the shaker in the peptone glycerol media, with and without added phosphate. The microscopic appearance of the cells in both media was strikingly similar to that of the Hy strain of *S. marcescens*. The cytoplasm of the cells grown in the low phosphate medium was intensely stained by Schiff's reagent following treatment with periodate, whereas that of the cells grown with added phosphate showed only a trace of pink.

Therefore our results with *S. marcescens* are entirely in agreement with those obtained by Duguid with *Aerobacter aerogenes:* when growth was checked by low concentrations of nitrogen or of phosphate, the cells elaborated capsular material and assumed a "nuclear" appearance. This was particularly evident when the cultures were grown on a shaker. In addition, it was found that the cytoplasm of these "nuclear" cells, which had lost the ability to retain simple basic dyes, gave a positive reaction for polysaccharides when exposed to Schiff's reagent after treatment with periodate. It is of interest to us that accumulation of the pigment prodigiosin, a pyrrole derivative (Wrede and Rothhaas: Z. physiol. Chem., **215**, 67; **219**, 267, 1933), paralleled the elaboration of polysaccharide in these cells.

ON THE NAMING OF TWO ANTIBIOTICS FROM MEMBERS OF THE BACILLUS CIRCULANS GROUP: CIRCULIN AND POLYPEPTIN

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At the time a paper entitled "Circulin, an antibiotic from a member of the *Bacillus circulans* group" appeared (McLeod: J. Bact., **56**, 749, 1948), a manuscript describing "Circulin, an antibiotic from an organism resembling *Bacillus circulans*" was in press (Murray *et al.*: J. Bact., **57**, 305, 1949). From the

description of the antibiotics it became appafent that the two substances, although given the same name, differed greatly from each other in chemical and biological properties.

By mutual agreement between the groups involved it was decided that the name *circulin* would be given to the Purdue antibiotic, notwithstanding the fact that the V. D. Research Laboratory group had been first to use this name in a scientific journal. Isolate Q-19, the organism producing this antibiotic, resembles *Bacillus circulans* more closely than does *Bacillus krzemieniewski* M-14, the organism producing the substance discovered at the V. D. Research Laboratory. It was felt that for that reason the name circulin was more appropriate for the product from strain Q-19. Furthermore, the name circulin had been used for the Purdue antibiotic for some time in the distribution of the material to various laboratories for clinical and chemical studies.

The Purdue circulin is chemically and biologically very similar to the polymyxins that are produced by strains of *Bacillus polymyxa*, although it is not identical to polymyxin A, B, C, or D (Murray *et al.*: *loc cit.*; Peterson and Reineke: *unpublished*). Circulin may eventually be classified with the polymyxins or other antibacterial polypeptides when the chemistry of these substances becomes better known. The term circulin could then still be used as a specific name for the compound from *Bacillus circulans*.

It is also proposed that the antibiotic called circulin by the V. D. Research Laboratory group be called *polypeptin*. This name is suggested because the active substance is a crystalline basic polypeptide (Howell: Federation Proc., 8, 208, 1949).

THE ABILITY OF SOIL MICROORGANISMS TO DECOMPOSE STEROIDS

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By means of enrichment culture techniques Turfitt (1944) demonstrated that the breakdown of cholesterol in soils was performed primarily by nocardiae among which *Nocardia erythropolis* was particularly efficient. Other actinomycetes, bacteria, and fungi were less effective or completely inactive. Similar enrichment culture studies with steroids other than cholesterol likewise produced nocardiae (Turfitt, 1947).

These findings, however, are perplexing since an ability to transform steroids oxidatively or reductively has been established for such diverse forms as *Alcaligenes faecalis* (Schmidt and Hughes, 1942; Schmidt, Hughes, Green, and Cooper, 1942; Hughes and Schmidt, 1942; Hoehn, Schmidt, and Hughes, 1944), *Azotobacter* (Horvath and Kramli, 1947), *Corynebacterium mediolanum* (Mamoli, 1944), *Eberthella typhosa* (Exner and Heyrovsky, 1908; Licht, 1924), *Escherichia coli* (Bondi, 1908; Exner and Heyrovsky, 1908; Fukui, 1937; Licht, 1924; Sihu, 1938), *Flavobacterium androstenedionicum* (Ercoli and Molina, 1944), *Flavobacterium carbonilicum* (Molina and Ercoli, 1944), *Flavobacterium dehydrogenans* (Arnaudi, 1942), *Klebsiella pneumoniae* (Exner and Heyrovsky, 1908), *Micromonospora* (Erikson, 1941), *Mycobacterium lacticola* (Turfitt, 1944), *Proteus vulgaris* (Licht, 1924; Mori, 1935), *Pseudomonas aeruginosa* (Licht, 1924), and others (Meyerstein, 1907; Pincus and Pearlman, 1943; Zimmermann and May, 1944).

It was, therefore, not wholly surprising when results recently obtained in this laboratory indicated the existence in nature of certain gram-negative bacteria, apparently abundant in number and of widespread distribution, that are capable of oxidizing a variety of compounds of the steroid group.

MATERIALS AND METHODS

For isolation, enrichment cultures were set up containing 0.06 to 0.1 per cent cholesterol as the sole organic constituent in an original medium (table 1); Turfitt's two media (1947), one for molds and the other for bacteria and actinomycetes, were also employed. Ether solutions of steroids and certain other cyclic compounds were added to the warm autoclaved basal medium, which was then inoculated after volatilization of the organic solvent. Neutralized bile acid and digitonin media were sterilized by etherization. In the case of benzene and cyclo-

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hexane, tubes of inoculated mineral medium were incubated in jars containing pools of the substances, the vapors of which distilled over into the cultures. Incubation was at 28 to 30 C.

Inocula included a tomato field soil, cornfield soil, a petroleum-saturated soil and a petroleum-free sample collected near an oil well, forest soil, peat, lake sediment, leaf compost, stable manure, and rabbit droppings. Fresh medium was inoculated from cultures exhibiting growth as a pellicle or turbidity. Any transfers that grew were in turn once more passaged. Agar plates were then streaked from obviously nonsterile third series cultures and pure type colony isolates picked. Primary enrichment cultures with no growth were discarded after 6 to 8

TABLE 1

Original medium

COMPOSITION		GROWTH FACTOR SUPPLEMENT EMPLOYED FOR ENRICHMENT CULTURES ONLY	
	%		*mg %*
Cholesterol...................	0.06–0.1	DL-Tryptophan	2.0
KH$_2$PO$_4$..	0.1	DL-Methionine........	1.0
NH$_4$NO$_3$	0.2	Uracil..	0.8
MgSO$_4$·7H$_2$O	0.02	Hypoxanthine...........	0.8
Fe*.....	1.0 mg	Nicotinic acid.	0.02
Ca...	0.05 mg	Thiamine	0.02
B..... . . ..,. ..	0.02 mg	Ca-pantothenate........ .	0.02
Co.....	0.01 mg	Pyridoxine......	0.02
Cu	0.001 mg	Riboflavin.	0.01
Mn......	0.0001 mg	Folic acid...	0.005
Mo...	0.01 mg	*p*-Aminobenzoic acid.........	0.01
Zn..	0.02 mg	Biotin......	0.0001
Distilled water			
pH 6.2–69			

* One mg per cent Fe supplied to enrichment cultures as dilute humate solution prepared according to Horner *et al.* (1934); 0.35 mg per cent Fe as FeCl$_3$ or FeSO$_4$·7H$_2$O employed for subsequent studies.

weeks; several of these that were passaged despite absence of visible growth yielded negative transfers.

Cholesterol determinations were carried out colorimetrically on duplicate or triplicate aliquots of the dry chloroform extracts of the culture media by the method of Schoenheimer and Sperry (1934). A preliminary digitonin precipitation, although used by Turfitt (1944), was found to be unnecessary and was, therefore, not employed.

EXPERIMENTAL RESULTS

From the 10 source materials investigated, 27 cultures representing different colony isolates were obtained with the original enrichment medium. Of these, 22 readily developed on cholesterol in agitated and static cultures as indicated by turbidity and lowering of pH. As the growth factor supplement was, with

few questionable exceptions, of no value, it was dispensed with for subsequent work. Of the 22 organisms, 19 appeared morphologically and culturally related.

These 19 organisms were all obligately aerobic, gram-negative, nonmotile rods, 0.6 to 0.8 μ by 2.5 to 3.5 μ, occurring singly, in pairs, or in short chains. On potato and nutrient agar slants, growth was generally luxuriant, moist, glistening, nonmucoid, and eventually a light pink. All but 5 strains grew on nutrient agar at 37 C. In 6 days, 8 cultures alkalinized litmus milk, which was unaltered by the others. All developed pellicles in nutrient broth and in synthetic mineral, NH_4NO_3 broth with glycerol or succinate. No strain hemolyzed blood or produced indole. Gelatin was liquefied by only 2 cultures.

All 22 strains were characterized as bacteria; not a single actinomycete or fungus was obtained. Moreover, every one of the 10 widely different soils, manures, and composts, ranging from pH 3.9 to pH 7.5, was found to harbor cholesterol-oxidizing bacteria. Turfitt's two media yielded only a single cholesterol utilizer, a bacterium isolated with the mold medium.

Because of inhibitory properties of bile acids and antibacterial effects of cholesterol, ergosterol, and certain of their derivatives (Raab, 1946; Squire and Squire, 1948), it was thought that this might be a significant factor in the enrichment medium. However, toxicity tests (table 2 together with other data not reported here) revealed that cholesterol, cholesteryl acetate, and cholesteryl palmitate were not inimical to the growth of a wide variety of bacteria, actinomycetes, and fungi. Ergosterol, on the other hand, inhibited 3 of 5 test bacteria, and 8 of 10 actinomycetes, but none of 5 molds. Consequently, the original cholesterol medium appeared to be nonselective, at least from the point of view of toxicity of the steroid. Ergosterol inhibition of actinomycetes but not fungi may perhaps explain why the latter but not the former organisms grew on this steroid as sole substrate. But this does not explain why 0.1 per cent cholesterol, which was not inhibitory, was utilized by actinomycetes but not fungi.

For more detailed studies on the decomposition of cholesterol, bacterium no. 5, isolated from fresh rabbit droppings, was selected. This particular culture was the most active user of cholesterol in both static and agitated cultures. The rate at which it oxidized cholesterol is shown in table 3.

As anticipated, the disappearance of cholesterol was more rapid under shaking than under static conditions, so that stabilizing the pH in a favorable range by means of $CaCO_3$ permitted oxidation of more steroid. On the other hand, $CaCO_3$ did not exert any marked beneficial effect in stationary cultures where growth was slower, although here the pH after 20 days had fallen to 4.55 as compared to pH 3.89 for 7-day-old shake cultures. Cholesterol was also oxidized in the presence of tryptone, but at a lower rate than when the steroid was the sole substrate available.

In order to compare bacterium no. 5 with *Nocardia erythropolis*, the species reported most active by Turfitt, the two organisms were inoculated into basal medium containing 0.1 per cent of various steroids as substrates. The results (table 4) show that the *Nocardia* developed on only 5 whereas the bacterium grew on 9 of the 14 compounds tested. Extending the period of observation beyond

TABLE 2

Cholesterol and ergosterol: utilization and toxicity

	CHOLESTEROL			ERGOSTEROL		
	Toxic-ity*	Utilization†		Toxic-ity*	Utilization†	
		Age	pH		Age	pH
		days			*days*	
Bacillus mycoides..........	+			0		
Bacillus subtilis...	+			0		
Escherichia coli	+			+		
Serratia marcescens....	+			+		
Staphylococcus aureus	+			0		
Streptomyces antibioticus...	+	8	6.50	+	6	6.15
Streptomyces griseus	+	8	6.50	0	6	6.20
Streptomyces lavendulae .. .	+	8	6.55	0	6	6.25
Micromonospora sp. . .	+	8	6.45	0	6	6.30
Mycobacterium smegmatis .. .	+	8	5.85	0	6	6.00
Nocardia corallina .	+	8	6.35			
Nocardia erythropolis... . .	+	8	3.80	+	6	4.20
Nocardia farcinica, no. 1 .		8	6.50		6	6.30
Nocardia farcinica, no. 2		8	3.90		6	5.50
Nocardia gardneri . . .	+	8	6.50	0	6	6.30
Nocardia maculata.. . .		8	6.55		6	6.20
Nocardia mexicanus . . .	+	8	4.20	0	6	6.20
Nocardia paraffinae....		8	4.20		6	6.20
Nocardia polychromogenes...		8	5.55		6	6.25
Nocardia salmonicolor		8	6.35		6	6.30
Nocardia sp., no. 3	+			0		
Nocardia sp., no. 4......	+			0		
Aspergillus fumigatus... .	+	12	6.70	+	6	6.00
Chaetomium cochliodes..	+	12	6.70	+	6	6.20
Gliocladium sp...	+	12	6.70	+	6	5.95
Neurospora crassa		12	6.99			
Ophiostoma catanianum.		12	6.71			
Penicillium notatum....	+	12	6.70	+	4	3.65
Phycomyces sp...	+	12	6.68	+	6	6.25
Torula sp......		12	6.70		6	5.40
Verticillium sp...		12	6.67		6	6.00
Uninoculated control.....		12	6.90		6	6.30

     * + = growth, 0 = no growth in original medium (table 1) supplemented with 0.2 per cent tryptone and 0.1 per cent steroid. Static cultures. Observed on third to twelfth day, whenever steroid-free controls exhibited positive growth.
     † Original medium plus 0.1 per cent steroid. Shake cultures.

the twelfth day would not have altered results since by that time flasks without visible growth contained no viable cells, as determined by plate counts for the bacterium and agar streaks for the actinomycete. This suggests that lower concentrations of the steroids might not have been inhibitory, and under such

TABLE 3

Rate of cholesterol utilization by bacterium no. 5

AGE	±1.0% CaCO₃		NO CaCO₃		CONTROL	
	pH	Cholesterol	pH	Cholesterol	pH	Cholesterol
Shake cultures						
days		*mg %*		*mg %*		*mg %*
1	7.47	65.6	5.50	75.8	6.10	72.0
	(7.47)*	(60.0)				
3	7.77	25.6	4.22	—	6.10	76.0
	(8.07)	(23.0)				
7	7.88	14.0	3.89	47.0	6.05	72.0
	(8.30)	(23.0)				
Static cultures						
1	7.49	72.0	5.80	89.2	6.10	76.0
3	7.72	63.2	5.55	60.0	—	—
7	8.20	58.4	5.37	66.2	6.05	74.4
13	8.17	27.0	4.82	10.8	6.30	78.0
20	7.90	12.2	4.55	10.4	6.30	78.0

* Results obtained with 0.1 per cent tryptone present.

TABLE 4

Utilization of steroids by bacterium no. 5 and Nocardia erythropolis

0.1% STEROID	BACTERIUM NO.			N. ERYTHROPOLIS			UNINOC-ULATED CON-TROL, pH
	Age*	pH	Viable cells per ml × 10⁶†	Age	pH	Relative growth	
Static cultures							
	days			*days*			
Cholesterol	5	6.30	91	7	6.50	++	6.90
Cholesteryl acetate.......	9	4.51	424	12	—	0	6.62
Cholesteryl palmitate....	12	—	0	7	6.35	++	7.00
Ergosterol.....	12	—	0	12	—	0	6.90
Cholic acid...	5	6.50	4	12	—	0	6.95
Desoxycholic acid.......	12	—	0	12	—	0	6.80
Lithocholic acid.....	4	4.70	306	12	—	0	6.95
Hyodesoxycholic acid....	12	—	0	12	—	0	6.75
Estriol..	4	4.60	262	12	—	0	6.60
Estradiol....	4	5.10	272	12	—	0	6.60
Testosterone........	9	4.41	440	11	3.95	++++	6.60
Progesterone........	12	—	0	7	3.75	++++	6.70
Dehydroisoandrosterone.....	4	5.50	1	7	6.25	++	6.65
Digitonin..............................	11	5.98	10	12	—	0	6.90

* Observed when positive growth appeared
† Inoculum: 2.4 × 10³ cells per ml.

conditions the range of compounds utilized might have been greater for one or both organisms.

It is interesting that the bacterium was killed by cholesteryl palmitate and *N. erythropolis* by both cholesteryl acetate and ergosterol. Previously, with respect to the toxicity tests reported in table 2, it had been pointed out that cholesterol, cholesteryl acetate, and cholesteryl palmitate did not inhibit growth of any of the bacteria, actinomycetes, or fungi tested, nor was ergosterol toxic to *N. erythropolis*. In those tests, however, tryptone was available in addition to the steroid, whereas in the present experiment steroids were the sole substrates.

Of the 19 cholesterol utilizers, only one grew with 0.1 per cent ergosterol. Most strains developed to a greater or less extent on 0.05 per cent desoxycholic acid, cholesteryl acetate, and cholesteryl palmitate. Anthracene, 1,2-benzanthracene, and phenanthrene at 0.1 per cent levels and cyclohexane and benzene vapors were generally unsatisfactory. At 0.1 per cent, stilbestrol, reported to be antibacterial by Brownlee *et al.* (1943), allowed no growth.

<div align="center">DISCUSSION</div>

These and Turfitt's results are perplexing for several reasons: (1) Turfitt's data indicate that nocardiae are the organisms predominantly responsible for the disappearance of steroids in soils, whereas the present investigations uncovered solely gram-negative bacteria. Actually Turfitt (1947) did obtain some gram-negative bacteria as cholesterol oxidizers, but these constituted a minority group among the active nocardiae. (2) Although Turfitt (1944) had demonstrated that numerous stock cultures of known common nocardiae were capable of utilizing cholesterol, his 188 isolates comprised only 5 species (*N. erythropolis*, *N. aquosa*, *N. globerula*, *N. coeliaca*, and *N. restricta*). Analogously, in the present studies (table 2) *Mycobacterium smegmatis* and various soil nocardiae were found capable of developing on cholesterol, yet not a single one of these was isolated by the enrichment culture technique. (3) Although common soil fungi can grow on ergosterol (table 2), Turfitt's enrichment cultures with this steroid yielded largely nocardial forms but no molds. (4) With Turfitt's two media, very poor results were obtained, 21 of the 22 cholesterol oxidizers having been isolated with the original medium.

It is hardly conceivable that American and British soils vary so qualitatively in microbial content that this might be responsible for the disparate results of Turfitt and ourselves.

One might be tempted to consider the absence of *added* trace elements (Ca, B, Co, Cu, Mn, Mo, and Zn) as responsible for Turfitt's failure to isolate bacteria and fungi in light of such evidence as (1) the limited but suggestive findings of Hutner (1947) on the Ca requirement of certain gram-negative bacteria, (2) the work of Steinberg (1948) on trace element requirements of fungi, and (3) a probable medium deficiency according to Turfitt's own observation (1947) that repeated transfers of fungi on steroid media produced only "a few struggling hyphae on the surface" but never a definite mycelial mat. But Turfitt's original enrichments must have contained trace elements from the fairly heavy soil

inoculum. Consideration of the trace element supplement, however, cannot explain the absence of molds and actinomycetes among cholesterol oxidizers isolated in the present studies, nor the failure to obtain actinomycetes even with the medium successfully employed by Turfitt for that purpose.

By the agar streak test several strains of the cholesterol bacteria were not demonstrably antagonistic to a variety of fungi, bacteria, and actinomycetes (including *N. erythropolis*). Nor was 0.1 per cent cholesterol toxic to various actinomycetes, bacteria, and fungi. Growth factors were not required by Turfitt's cultures or by the bacteria isolated during the present investigations.

Consequently, an explanation for the above-mentioned discrepancies is at present lacking. It would appear, however, that either Turfitt's data or those herein reported, if considered separately, are too restricted to allow one to generalize on the nature of steroid decomposers among the soil microflora. It is certain that in addition to nocardiae there are widely distributed and apparently abundant gram-negative, nonmotile, rod-shaped eubacteria capable of decomposing steroids. Moreover, limited work with fungi indicates that under suitable conditions common soil molds are capable of oxidizing ergosterol.

SUMMARY

By means of enrichment cultures with cholesterol as substrate only nonmotile, gram-negative, rod-shaped eubacteria were isolated from various soils and manures and from compost, peat, and lake sediment ranging in pH from 3.9 to 7.5. No actinomycetes or molds were isolated, although several of the former from the culture collection were shown to be fully capable of growth in the mineral-cholesterol medium. As is discussed, this is, for reasons at present unknown, not in agreement with the findings of Turfitt, who isolated nocardiae as the predominant steroid oxidizers in soils.

Comparative studies with *Nocardia erythropolis*, Turfitt's most active species, and with a bacterium isolated during the present investigations revealed that the actinomycete attacked fewer steroids than did the bacterium under given test conditions.

Limited work with fungi revealed that in shake cultures with 0.1 per cent steroid, several common soil forms developed on ergosterol but not on cholesterol, neither being toxic at this level.

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A SIMPLIFIED LIQUID CULTURE MEDIUM FOR THE GROWTH OF HEMOPHILUS PERTUSSIS

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Hornibrook (1939) described a liquid medium for the propagation of *Hemophilus pertussis* having a casein hydrolyzate base to which were added various salts, starch, cysteine, and nicotinic acid and which was capable of growing recently isolated strains. Because of the potential advantages of such a medium, it has been studied by many investigators. Their work has confirmed Hornibrook's original observations but it has also revealed several disadvantages in the medium. Modifications of the original formula stated to prevent precipitation, simplify preparation, decrease the granularity of growth, and increase the final bacterial count have been suggested by Verwey and Sage (1945), Wilson (1945), Farrell and Taylor (1945), and Cohen and Wheeler (1946).

It is the purpose of this report to describe further the medium that has been developed in our laboratory and to define the cultural conditions that seem optimal for the growth of *H. pertussis* in this medium. Some observations concerning the antigenic properties of the liquid medium cultures are included.

EXPERIMENTAL METHODS AND RESULTS

Culture medium and growth studies. In table 1 the general formula of our medium has been compared with those of others. Quantities have been expressed in grams or milliliters per liter of culture medium, and the figures for casein hydrolyzate and sodium chloride have been calculated with recognition of the fact that "casamino" acids (Difco technical) contain approximately 40 per cent sodium chloride. Although, for purposes of simplicity in presentation, the phosphate and sulfur sources have not been specified, all formulae made use of sodium or potassium phosphate and cysteine, cystine, or glutathione.

These modifications of the Hornibrook formula have certain features in common. Calcium chloride has been reduced in amount or eliminated to prevent the troublesome precipitation of calcium phosphate that occurred frequently in the original medium. Except in the Wilson formula, the quantity of phosphate has been increased to buffer the medium against the strong tendency of *H. pertussis* to cause an alkaline reaction. In addition, the concentrations of casein hydrolyzate are increased somewhat except in the formula of Cohen and Wheeler. The formula in this paper is given specifically in table 2 and contains balanced changes representing all of these modifications. All ingredients may be added before sterilization. It is to be noted that the formula makes use of no accessory growth factors other than those supplied by the casein hydrolyzate itself. Furthermore, it has been found that no growth stimulation resulted from the addition of the

accessory growth factors present in blood cell extract or liver extract. This result was different from the earlier report of Verwey and Sage (1945) in which blood cell extract was shown to produce a considerable improvement in final bacterial

TABLE 1

Culture medium composition expressed in grams per liter

	HORNI-BROOK	WILSON	FARRELL AND TAYLOR	COHEN AND WHEELER*	VERWEY ET AL.
Casein hydrolyzate...........	7.0	10.0	10.0	6.0	9.4
NaCl.......................	5.0	5.0	5.0	6.5	4.6
KCl.......................	0.2	0.2	0.2	—	0.2
CaCl₂......................	0.2	0.002	—	0.1	—
MgCl₂·6H₂O................	0.1	0.025†	—	0.4	0.1
Na₂CO₃....................	0.5	0.4	—	—	—
Phosphate.................	0.25	0.25	0.4	0.5	0.5
Starch.....................	1.0	1.0	1.0	1.5	1.0
Nicotinic acid....	0.01	—	0.001	—	0.02
Sulfur source.....	0.01	0.01	0.022	0.025	0.01
Accessory factors..	—	Liver ext. 0.4 ml	Liver ext. 20 ml	Yeast dial. 50 ml	—

* FeSO₄·7H₂O, 0.01; CuSO₄·5H₂O, 0.005, additional ingredients.
† MgCl₂·8H₂O.

TABLE 2

Liquid medium for the growth of Hemophilus pertussis

Distilled water..	1 liter
Difco "casamino" acids technical grade...........	14 grams
KCl*...	0.2 gram
KH₂PO₄..	0.5 gram
MgCl₂·6H₂O..	0.1 gram
Nicotinic acid.....................................	0.02 gram
L-Cystine†..................................	0.01 gram
Starch‡	1 gram

The pH is adjusted to 6.8 and the medium is then autoclaved 10 minutes at 15 pounds to get material into solution. The pH is rechecked and should be from pH 6.8 to 6.9. The medium is bottled and sterilized at 15 pounds for 15 minutes. This medium will keep from 4 to 8 months.

* Experiments have shown that the inclusion of KCl usually is not necessary.
† Glutathione may be substituted for L-cystine in the same amount.
‡ Starch granules are first thoroughly wet with cold water. This suspension is then added to hot water and the mixture brought to a "rolling" boil. The resulting solution is added to the rest of the medium.

density. The reason for this difference may be in the nature of the casein hydrolyzate supplied by the Digestive Ferments Company. Lots purchased in 1941, 1942, and part of 1943 were deficient in some factor that has appeared regularly in subsequent batches. This deficiency was overcome by blood cell extract. The identity of this factor is not known at present, although considerable information

tending to eliminate from consideration many of the recognized accessory growth substances has been obtained.

In addition to the ingredients of the culture medium, cultural conditions including inoculum size, time of incubation, surface-volume relationships, and agitation have a profound effect on the growth rate and final density of *H. pertussis* cultures grown in liquid media. Therefore, it is important to define these conditions in evaluating the results of any cultural procedure. Table 3 describes the cultural conditions employed in our laboratory and compares them with those of other workers. The sizes of the inocula that were used were not specified completely by Wilson or by Farrell and Taylor, so these have been estimated from the stated inoculum volumes and the reported densities usually obtained.

It is believed that the favorable results that have been obtained with our procedure are attributable to a combination of several factors. During the early

TABLE 3

Cultural conditions for the growth of H. pertussis in liquid media

	HORNIBROOK	WILSON	FARRELL AND TAYLOR	COHEN AND WHEELER	VERWEY ET AL.
Incubation temp.	37 C	37 C	37 C	35 C	37 C
Incubation time, in days	2–4	4–5	3	3	2–3
Agitation	Intermittent	Intermittent	Continuous	Intermittent	Continuous
Inoculum per liter	Unspecified	2,500 B	20 B	750–1,000 B	400–1,000 B
pH	7.4	7.0	7.1	7.2–7.3	6.8
Density per ml	15 B+*	15 B+*	15 B+*	10–15 B	20–50 B

* No maximum density or range of densities given.

work with Hornibrook's medium it became apparent that the development of an alkaline reaction in the cultures was a growth-limiting condition. When the buffering power of the medium was increased, further additions of casein hydrolyzate and nicotinic acid resulted in increased density. It was observed also that the increase in pH that occurred was related both to the bacterial density and to the time of incubation. Thus, 72-hour cultures that had grown to a specified density were always more alkaline than the 48-hour cultures at the same density. This observation suggested that efforts should be made to produce rapid growth so that maximal multiplication would take place before the medium had become strongly alkaline. It was found that, through the use of relatively large inocula of 400 to 1,000 billion organisms per liter, greater final densities could be produced and the cultures reached their maximal values more quickly. When these procedures were combined with the use of constant agitation on a shaking machine operating at 120 strokes a minute on a traverse of approximately 8 mm, the most satisfactory results were obtained. Cultures that were incubated in 250-ml quantities contained in liter Blake bottles placed horizontally on the

shaking machine grew regularly to densities between 20 and 50 billion organisms per ml in 48 hours.

Figure 1 shows the frequency of occurrence of various bacterial turbidities at 48 and 72 hours in a large series of cultures that have accumulated during the last three years. It can be seen that the 48-hour cultures show a marked peak between 20 and 30 billion, but almost half are above 30 billion. The maximal density found was 55 billion per ml at 48 hours and 77 billion at 72 hours.

This medium has been tried for the growth of 13 strains of *H. pertussis* that were demonstrated to be typically smooth. All but one of these strains grew readily to densities within the range previously mentioned. No adaptation seemed
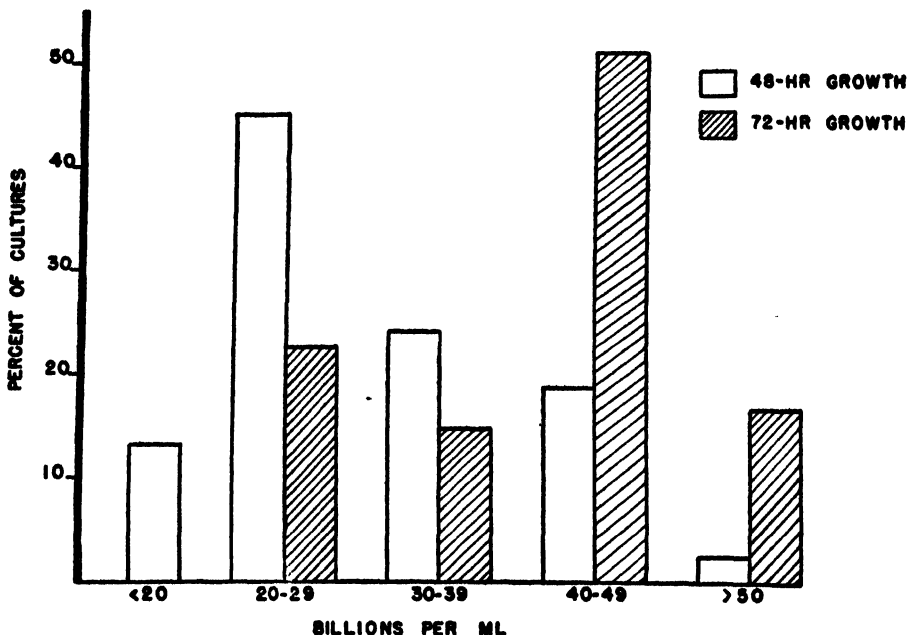
Figure 1. Density of *H. pertussis* cultures grown in liquid medium.

to be required in this medium since first subcultures from Bordet Gengou agar grow as rapidly as, and had densities equal to, subsequent subcultures.

Microscopic examinations of stained preparations indicated that organisms grown in this medium were short coccobacillary forms very similar in appearance to those harvested from Bordet Gengou agar. Filaments and clumps were relatively infrequent. Repeated consecutive subculture through as many as 19 passages in this medium caused no obvious change in cellular or colonial morphology, and the cultures remained as smooth, phase I cultures. A strain of *H. pertussis* having an intracerebral mouse virulence of 200 to 400 organisms was carried through nine successive subcultures in the liquid medium without appreciable loss of infectivity.

Recent studies have indicated that glutathione may be a considerably more

effective sulfur compound than is L-cystine when it is used in similar amounts. Although the trials of the medium containing glutathione have not been as extensive as those employing L-cystine, the results indicate that glutathione causes more rapid growth and higher densities after 48 hours of incubation. A comparison of the results obtained with these two sulfhydryl compounds is given in figure 2 in which density figures obtained with strains 2227 and 2231 have been combined. These figures were collected from a series of parallel cultures from Bordet Gengou agar inocula.
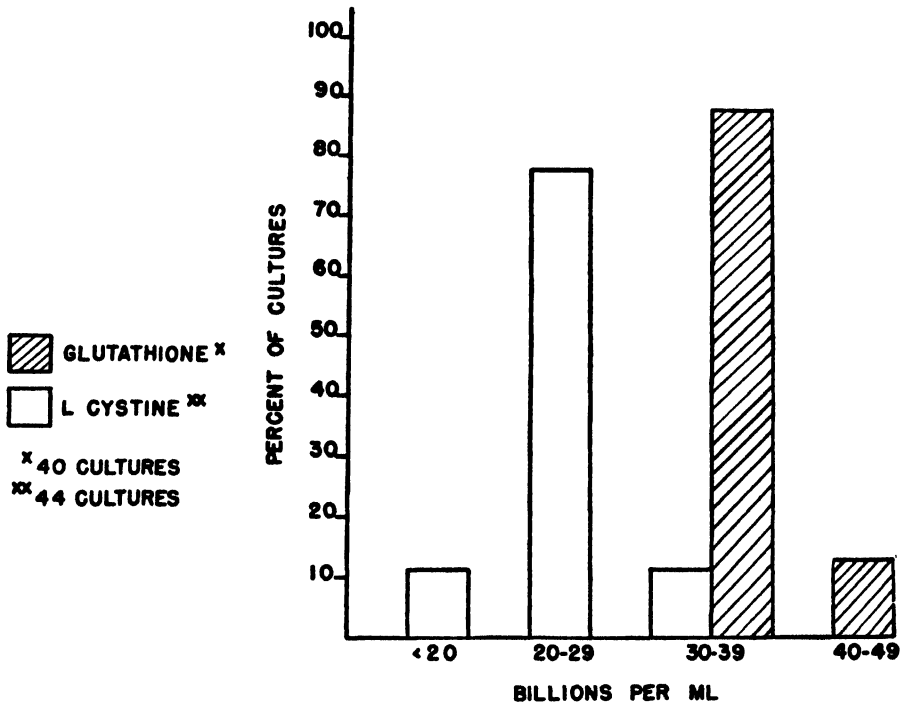
Figure 2. Density of *H. pertussis* cultures grown 48 hours in liquid medium containing L-cystine or glutathione.

Vaccine antigenicity. Pertussis cultures grown in a liquid medium and killed by the addition of sodium ethylmercuri-thiosalicylate ("merthiolate") or phenol are outstandingly suitable for the preparation of immunizing vaccines. The whole culture (properly killed and standardized) is safe for injection as a vaccine since it contains no foreign protein other than that contributed by the organisms themselves. Such a vaccine has been in use for more than four years, and over this time there has been reported no instance of sensitization that could be attributed to the medium.

Numerous vaccine preparations killed with either 0.5 per cent phenol or 1:10,000 sodium ethylmercuri-thiosalicylate have been tested for antigenicity. These vaccines when tested by the protection test methods described by Pittman (1946) or Kendrick *et al.* (1947) appear to be comparable to vaccines prepared

from organisms grown on Bordet Gengou agar. Also, they have been found to meet the standards for pertussis vaccine established by the National Institute of Health of the United States Public Health Service. Evidence accumulated in this laboratory suggests that the neutralization of the relatively high final alkalinity of liquid cultures is desirable to maintain the antigenicity of the cells during long periods of storage at refrigerator temperatures.

Solid medium studies. The liquid medium described above has been converted to a solid medium by the addition of 1.5 to 2.0 per cent agar. Only traces of growth appear following heavy inoculation. However, when human blood cells in saline equivalent to 10 to 20 per cent whole blood are added, good growth

TABLE 4

Comparative colony counts on Bordet Gengou and casein hydrolyzate blood agar

	72 HOURS			96 HOURS			120 HOURS		
	Casein hydrolyzate blood agar	BG I*	BG II†	Casein hydrolyzate blood agar	BG I*	BG II†	Casein hydrolyzate blood agar	BG I*	BG II†
Strain 2753	100	18	86	120	70	88	129	106	93
	99	23	80	115	65	95	115	115	95
	90	25	60	128	67	65	128	110	65
	96	20	83	116	47	83	116	99	83
	84	16	86	100	50	96	106	80	96
	88	10	60	120	48	73	120	110	73
Strain 2227	179	0	173	202	0	195	214	170	195
	85	43	66	124	95	86	132	107	108
Strain 2231	90	41	69	120	111	89	128	113	113
	81	45	63	129	79	83	137	101	103

Seed: 0.1 ml containing 200 organisms by turbidity estimation was spread on the surface of agar plates.

* Bordet Gengou agar prepared in the laboratory.
† Difco Bordet Gengou agar.

develops within 24 hours. This medium was compared with Bordet Gengou agar by spreading 0.1-ml quantities of dilute suspensions of *H. pertussis* on the plates. These data are summarized in table 4, and it can be seen that the casein hydrolyzate blood agar induced the growth of a larger number of colonies in a shorter period of time than did either of the two Bordet Gengou agar media that were tested.

Casein hydrolyzate blood agar containing 0.2 units of penicillin per ml has received limited trial in the isolation of *H. pertussis* from nasopharyngeal swabs. The results of this work indicated that satisfactory isolations could be obtained and that the overgrowth of contaminants was no more extensive with this medium than with Bordet Gengou agar containing comparable quantities of penicillin.

DISCUSSION

The medium that is described in this communication represents a simplification and a balancing of the original formula of Hornibrook. It has resulted in further improvement of growth-promoting properties and the elimination of all substances of indefinite chemical composition with the exception of starch and casein hydrolyzate. In addition, the medium has been found to be of considerable value both in the production of *H. pertussis* vaccines and in the study of the metabolic and antigenic constituents of the organism (Verwey and Thiele, 1949). The use of this medium and the cultural conditions described have resulted in more rapid growth and greater final bacterial counts than have been reported previously.

It is of interest that, although the medium appeared to be entirely adequate in its liquid form, it could not support the growth of *H. pertussis* when it was solidified by the addition of agar. It is not known at present whether this situation is the result of some inhibitory effect of agar or of a peculiarity of the respiratory requirements of the organism. However, even though blood cells are required for growth, it is believed that this solid medium may be of considerable usefulness because of the simplicity of preparing it and because of its superior growth-promoting characteristics. More extensive trials than those so far conducted will be required before this casein hydrolyzate blood agar may be evaluated properly in the laboratory diagnosis of pertussis infection. However, the results obtained seem to indicate that the medium may be able to replace Bordet Gengou agar in all of its normal uses.

SUMMARY

A modification of Hornibrook's liquid casein hydrolyzate medium for the cultivation of *Hemophilus pertussis* has been described, and improved growth conditions have been defined. It was found that culture densities between 20 and 50 billion cells per ml could be obtained regularly within 48 hours, and that organisms grown in this medium were similar culturally to those grown on Bordet Gengou medium. These cultures were suitable for the preparation of vaccines of satisfactory antigenicity as measured by mouse protection tests.

The medium that was developed contains only ingredients that are readily available in a dry, stable form. It can be prepared also as a solid medium by the inclusion of agar. Ten to 20 per cent blood cells had to be added to the solid medium to obtain good growth, but when the blood cells were used, more colonies of *H. pertussis* could be grown with this medium from a dilute bacterial suspension than with either of the two Bordet Gengou agar preparations that were tested. Since these colonies appeared earlier and grew larger than those on the Bordet Gengou plates, it has been suggested that the casein hydrolyzate blood agar might be suitable for use in the laboratory diagnosis of pertussis infection.

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THE VITAMIN REQUIREMENTS OF LEUCONOSTOC FOR DEXTRAN SYNTHESIS

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To overcome variable yields in the synthesis of dextran from sucrose by *Leuconostoc*, addition to the media of materials such as raw beet sugar or molasses (Carruthers and Cooper, 1936), commercial maple syrup (Stacey and Youd, 1938), yeast extract or tomato juice (Daker and Stacey, 1939), raw cane sugar (Carlson and Stahly, 1939), aqueous extracts of waste sugar-refining charcoal (Stahly, 1943), raw fig (Bouillenne, 1938), and sterile *Staphylococcus aureus* filtrates (Carlson, 1939), as well as a mixed culture with *Saccharomyces cerevisiae* (Stacey, 1942), has been recommended. Since these observations were made concerning peptone media, it appeared that a necessary preliminary to the investigation of the stimulatory factor or factors present in these diverse materials was a study of the vitamin requirements of *Leuconostoc* in sucrose media of known composition. Several reports (Snell *et al.*, 1939; Gaines and Stahly, 1943; Johnson, 1945; Shankman *et al.*, 1947) were found on vitamin requirements of *Leuconostoc* for growth in chemically defined glucose media, but no polysaccharide is formed by the organisms from this sugar. Because we are primarily interested in the stimulatory substances contained in raw cane sugar, the present study was further necessitated by reports (Jackson and Macek, 1944; Hall *et al.*, 1947) on the presence of members of the B group of vitamins in raw sugar.

EXPERIMENTAL METHODS

Cultures. Three strains of *Leuconostoc mesenteroides* and three of *Leuconostoc dextranicum* obtained from G. L. Stahly, Ohio State University, were employed in various phases of the investigation. One other strain, *L. mesenteroides* 8042 (ATCC), was not found capable of forming dextran from sucrose under any conditions tried. We agree with Camien *et al.* (1947) that it probably should be classified as *Leuconostoc citrovorum*. The results reported here are those obtained with two strains of *L. mesenteroides*, 535 and 683, and with *L. dextranicum* 8086 (ATCC).

Media. The basal medium employed had the composition given in table 1. The sugars used were either glucose, fructose, or sucrose. The pH of the media was adjusted to neutrality. The media, distributed in 3-ml amounts into 12-by-100-mm tubes which were closed by cotton plugs, were sterilized 10 minutes at 15 pounds pressure.

Inocula. The cultures were carried on 2 per cent sucrose Difco nutrient broth, not fortified with added vitamins. After 36 hours' incubation at room temperature the cells were centrifuged from the medium, resuspended in sterile saline, and again separated by centrifugation. The cells were washed a second time by

this procedure, after which they were suspended in a volume of sterile saline equal to that of the medium in which they were grown. By means of a sterile syringe (20-gauge needle) one drop of this suspension was added to each tube of medium. The tubes were incubated in a cabinet equipped to maintain a constant temperature of 25 C. Upon completion of the experiment, growth was arrested by steaming the tubes for 20 minutes. Kitay and Snell (1948) have recently re-emphasized the influence of the size of the inoculum on the apparent vitamin requirements of lactic acid bacteria. In the present case the inoculum used was as small as was consistent with growth in the complete basal medium containing

TABLE 1

Composition of basal medium

	mg/L		mg/L
L-Glutamic acid	500	Xanthine	10
DL-Valine	500	Thymine	10
DL-Isoleucine	500	Nicotinic acid	1.0
DL-Leucine	500	Riboflavin	0.5
DL-Alanine	400	Thiamine chloride	0.5
DL-Methionine	400	Calcium pantothenate	0.5
L-Cysteine	400	Pyridoxal·HCl	0.2
L-Asparagine	200	Pyridoxamine·2HCl	0.2
L-Lysine·HCl·H$_2$O	200	p-Aminobenzoic acid	0.1
DL-Threonine	200	Folic acid	0.01
DL-Phenylalanine	200	Biotin	0.001
DL-Tryptophan	200	MgSO$_4$·7H$_2$O	100
DL-Serine	200	NaCl	100
Glycine	200	FeSO$_4$	10
DL-Aspartic acid	200	MnSO$_4$	10
L-Arginine·HCl	100	KH$_2$PO$_4$	500
L-Histidine·HCl·H$_2$O	100	K$_2$HPO$_4$	500
L-Tyrosine	100		g/L
L-Proline	100	NaOAc	25
Adenine sulfate	10	NH$_4$Cl	5
Guanine·HCl	10	Carbohydrate	100
Uracil	10		

cp sucrose, glucose, or fructose. Inocula half as concentrated often failed to grow in the cp sugar media, although growth resulted consistently from the diluted inoculum when raw cane sugar was used as the carbohydrate source.

Analytical methods. The rate of growth of the organisms was measured in terms of acid production by titrating each tube of medium with standard alkali, bromthymol blue being used as indicator. Dextran yields were determined by gravimetric measurement of the amount of polysaccharide formed. Tared test tubes were employed, 5 ml of a 60 per cent ethanol and 40 per cent glacial acetic acid mixture being added to the fermented medium upon completion of incubation. The solutions were mixed and the tubes corked and centrifuged in an angle head for 20 minutes at 2,000 rpm. The supernatant fluid was decanted, the precipitated polysaccharide resuspended in 5 ml absolute ethanol, and the dextran

again separated by centrifugation. The tubes were thoroughly drained and then dried in an 80 C oven to constant weight. Dextran yields were calculated from the relationship $nC_{12}H_{22}O_{11} \rightarrow (C_6H_{10}O_5)_n$ and expressed as percentage of the theoretical yield corresponding to complete conversion of the glucose half of the molecule into the polysaccharide. Applied to known solutions of the polysaccharide, this method of treatment resulted in a nearly quantitative recovery of dextran. The same procedure was used on uninoculated control tubes and on actively growing cultures in glucose medium, in which no polysaccharide is formed; negligible amounts of materials were precipitated in these cases. All measurements of acid production and dextran yields were made either in duplicate or triplicate.

TABLE 2

Effect of vitamin omission on acid production and dextran yield in sucrose medium

VITAMIN OMITTED FROM MEDIUM	ACID PRODUCTION*			DEXTRAN YIELD		
	L. mes. 683	L. mes. 535	L. dex. 8086	L mes. 683	L. mes. 535	L. dex. 8086
	ml	ml	ml	%	%	%
None..	8.4	4.8	3.7	59	45	6
Folic acid..	1.4	5.5	0.3	20	50	†
PAB...	7.5	4.5	3.8	61	51	8
Riboflavin...	9.1	4.8	0.1	61	49	†
Thiamine...	0.2	0.2	0.1	†	†	†
Pyridoxal, pyridoxamine......	7.3	5.5	3.5	61	47	7
Pantothenic acid... . ..	2.3	1.2	0.1	21	5	†
Nicotinic acid.	0.5	0.4	0.3	3	3	1
Biotin..	9.1	4.5	4.3	64	49	9
Folic acid, PAB						
Riboflavin............................	1.4	3.4	0.0	21	43	†
Folic acid, PAB....	1.3	3.9	0.2	19	46	†
Riboflavin, PAB.....	9.4	5.3	0.1	59	50	†
Riboflavin, folic acid..	1.4	4.8	0.2	18	43	†

* In this and subsequent tables, as ml 0.01 N NaOH used per ml of medium.

† Less than 1 per cent.

RESULTS AND DISCUSSION

In table 2 are presented data for acid production and dextran synthesis in the basal medium containing 10 per cent cp sucrose. Corresponding data on acid production from fructose and from glucose are given in table 3. All the data for these tables were collected at one time in order to eliminate variations due to factors such as the size of the inoculum and the metabolic activity of the organisms. With all three sugars the results reported for acid production are somewhat lower than those frequently given (Shankman *et al.*, 1947), a fact traceable mainly to the incubation temperature employed (25 C), the time of incubation (48 hours), and the moderate inoculum used. Preliminary experiments indicated that dextran synthesis proceeded at a maximum rate at 25 C; polysaccharide

formation decreased as the temperature increased and very little polymer was obtained at 37 C. Hehre and Sugg (1942) have reported similar data for the effect of temperature on preparations of the dextran-synthesizing enzyme.

The preference of *Leuconostoc* for sucrose, rather than its constituent monosaccharides, is shown in tables 2 and 3. The data further support the claim (Carlson, 1939; Leibowitz and Hestrin, 1945) for direct utilization of sucrose by *Leuconostoc*. Other instances of direct utilization of disaccharides by microorganisms have been reported (Wright, 1936; Doudoroff, 1943, 1945; Snell *et al.*, 1948).

For the three strains of *Leuconostoc* studied thiamine, nicotinic acid, and pantothenic acid were essential in all three carbohydrate media. Folic acid was required

TABLE 3

Effect of vitamin omission on acid production in glucose or fructose medium

VITAMIN OMITTED FROM MEDIUM	ACID PRODUCTION GLUCOSE MEDIUM			ACID PRODUCTION FRUCTOSE MEDIUM		
	L. mes. 683	*L. mes.* 535	*L. dex.* 8086	*L. mes.* 683	*L. mes.* 535	*L. dex.* 8086
	ml	*ml*	*ml*	*ml*	*ml*	*ml*
None. . .	6.9	1.1	1.2	7.8	2.4	1.4
Folic acid..	1.4	0.8	0.5	1.7	2.2	0.6
PAB	6.8	0.9	1.1	7.7	2.1	1.1
Riboflavin.	7.3	1.0	0.2	7.3	1.7	0.4
Thiamine .	0.4	0.3	0.4	0.5	0.3	0.3
Pyridoxal, pyridoxamine	5.7	1.1	0.8	5.4	1.8	1.0
Pantothenic acid	0.8	0.5	0.4	1.4	0.6	0.3
Nicotinic acid	0.6	0.4	0.2	0.6	0.5	0.5
Biotin. ..	2.7	0.5	0.7	3.0	1.0	0.7
Folic acid, PAB Riboflavin..	1.2	0.6	0.3	1.2	0.9	0.3
Folic acid, PAB	1.3	0.6	0.3	0.8	0.6	0.5
Riboflavin, PAB.	6.5	0.7	0.1	5.9	0.9	0.8
Riboflavin, folic acid.	1.3	0.6	0.2	1.5	1.2	0.7

by *L. mesenteroides* 683 and *L. dextranicum* 8086, the latter organism also requiring riboflavin. In glucose and fructose media the omission of biotin caused a considerable reduction in growth with all three strains. In the sucrose medium, however, biotin appeared to be nonessential.

Data on vitamin requirements in glucose media of two of the strains employed, *L. mesenteroides* 535 and *L. dextranicum* 8086, are available in the literature. For the former organism Gaines and Stahly (1943) found nicotinic acid, pantothenic acid, thiamine, and biotin to be required. For both strains Shankman *et al.* (1947) report only nicotinic acid and pantothenic acid to be required. Since the composition of our basal medium is similar to that of Shankman and coworkers, it is probable that the more stringent vitamin requirements found in the present work are to be traced to the type of inoculum used. In particular, Gaines and Stahly (1943) found that avidin inactivation was necessary in order

to demonstrate a requirement for biotin in glucose medium by *L. mesenteroides* 535. Kitay and Snell (1948) have recently reported that the failure of Shankman *et al.* (1947) to demonstrate a biotin requirement for various lactic acid organisms was due to the heavy inocula used by these investigators. The result obtained here, that of a requirement for biotin in glucose and fructose media but apparent independence from this vitamin in sucrose medium, was also observed in studies involving avidin inactivation of the various media. This variation in vitamin requirement, dependent on the carbohydrate source, is being investigated further (Carlson and Whiteside-Carlson, 1949), especially in regard to its possible relationship to direct utilization of the disaccharide by *Leuconostoc*.

Shankman *et al.* (1947), noting the comparative rarity of inhibition by vitamins, pointed out that pyridoxine, pyridoxal, and pyridoxamine were slightly

TABLE 4

Effect of various concentrations of thiamine, nicotinic acid, and pantothenic acid on acid production and dextran yields by L. mesenteroides 683

VITAMIN ADDED		ACID		DEXTRAN	
		36 hr	48 hr	36 hr	48 hr
	µg/ml	*ml*	*ml*	*%*	*%*
Thiamine....	0.01	6.6	9.2	35	63
Thiamine....	0.1	6.9	10.0	36	55
Thiamine.	1.0	6.3	10.0	34	55
Thiamine....	10.0	5.1	8.8	25	52
Nicotinic acid...	0.01	1.1	1.2	11	15
Nicotinic acid.....	0.1	5.2	6.3	27	52
Nicotinic acid....	1.0	5.5	9.6	27	52
Nicotinic acid....	10.0	5.0	9.2	26	52
Ca-pantothenate..	0.01	4.7	8.2	21	43
Ca-pantothenate..	0.1	5.5	9.2	26	54
Ca-pantothenate...	1.0	6.1	9.8	27	54
Ca-pantothenate.....	10.0	4.8	9.0	29	55

inhibitory to acid production by *Lactobacillus brevis* and that *p*-aminobenzoic acid inhibited several other lactic acid organisms in the early stages of incubation. In the present investigation, especially when sucrose was the carbohydrate used, instances of inhibitory effects were noted with the various strains in the cases of riboflavin, biotin, folic acid, *p*-aminobenzoic acid, pyridoxal, and pyridoxamine.

The known presence of B vitamins in raw sugar and the probable presence of these materials in raw sugar concentrates necessitated the studies in table 2 on the effect of vitamin omission on dextran yields. From the results obtained it is seen that the elimination of any given vitamin had no adverse effect on either dextran yield or acid production unless that vitamin was essential for growth. These data, therefore, indicate that the stimulatory effect of raw sugar is not due to this substance's serving as a source of biotin, riboflavin, pyridoxal, pyridoxamine, folic acid, or *p*-aminobenzoic acid. To eliminate the possibility that raw sugar might function as an additional source of the essential vitamins,

nicotinic acid, pantothenic acid, and thiamine, the experiment reported in table 4 was performed. In this case the basal medium of table 1 was employed with the exception that the concentrations of these three vitamins were varied from 0.01 μg to 10 μg per ml. The data in table 4 indicate that different optima exist as regards dextran yield and acid production. When varying levels of the vitamins were used in conjunction with raw sugar, an inverse effect was noted, dextran yields with strain 683 ranging from 67 per cent with high concentrations of vitamins to 82 per cent with low concentrations. In any event, it is clear that increasing the concentrations of the vitamins over the values given in table 1 results in inhibiting effects on dextran yields, acid production, or both. The stimulation of acid and dextran production resulting from use of raw cane sugar, thus, is not due to the raw sugar serving as an additional source of the B vitamin used in these studies.

SUMMARY

Acid production from glucose and fructose and both acid production and dextran yields from sucrose were determined for three strains of *Leuconostoc* in a chemically defined medium. Thiamine, nicotinic acid, and pantothenic acid were required by all strains, the need for folic acid and riboflavin varying with the different strains. The requirement for biotin appeared to be a function of the carbohydrate used, this vitamin apparently being nonessential in a sucrose medium. The stimulatory effect of raw cane sugar could not be identified with any of the vitamins studied.

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STUDIES OF THE EFFECT OF PARA-AMINOBENZOIC ACID, FOLIC ACID, AND SULFANILAMIDE ON DEXTRAN SYNTHESIS BY LEUCONOSTOC[1]

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The factor stimulating dextran synthesis by *Leuconostoc* has been claimed by Stacey (1947) to be *p*-aminobenzoic acid. In studies on the vitamin requirements of *Leuconostoc* (Whiteside-Carlson and Carlson, 1949), it was reported that omission of *p*-aminobenzoic acid from the media did not adversely affect acid production or polysaccharide synthesis by the organisms. Since, however, the only identification of the substance in raw cane sugar, molasses, and the like that stimulates dextran synthesis by *Leuconostoc* has been that given by Stacey, this point was investigated further. The effect of sulfanilamide on polysaccharide synthesis by *Leuconostoc* was also studied, since Stacey suggested that the identity of the dextran synthesis factor as *p*-aminobenzoic acid was of interest in relation to the mode of action of the sulfonamides.

EXPERIMENTAL METHODS

Cultures. Two strains of *Leuconostoc mesenteroides*, 683 and 535, and two strains of *Leuconostoc dextranicum*, 8086 and elai, were employed in this investigation. The method of maintaining the cultures and preparing the inocula was the same as that described in the preceding paper.

Media. Two basal media were utilized. The first of these, an amino acid, purine, pyrimidine medium, had the composition given in the preceding paper. In the second type the amino acid mixture was replaced by acid-hydrolyzed (Landy and Dicken, 1942) Pfanstiehl H. P. casein, 5 g per L, fortified by cysteine 100 mg. per L, and tryptophan 100 mg per L. The purines, adenine and xanthine, were added in a concentration of 20 mg per L. As indicated, guanine, thymine, and uracil when used were present in a concentration of 10 mg per L. Vitamin concentrations in both basal media were the same except when noted otherwise. The sugar component used was present in a concentration of 50 g per L. Acid production and dextran yields were measured by the methods given in the preceding publication.

RESULTS AND DISCUSSION

Stacey (1947) did not specify whether *p*-aminobenzoic acid functioned directly in dextran synthesis or indirectly as a precursor of folic acid; hence we used both folic-acid-dependent and folic-acid-independent strains of *Leuconostoc*. In table 1 are presented data on the effect of a great variation in *p*-aminobenzoic acid concentration on acid formation and dextran synthesis by two folic-acid-inde-

pendent strains, *L. dextranicum* elai and *L. mesenteroides* 535. The results show that a thousandfold variation in *p*-aminobenzoic acid concentration affected dextran synthesis and acid production only slightly, and then in the direction of inhibition. Sulfanilamide, in a range of concentrations, sharply decreased acid production but affected dextran synthesis to a lesser extent. Both results minimize the effect of *p*-aminobenzoic acid on polysaccharide synthesis by these strains of *Leuconostoc*.

Table 2 presents the results of added folic acid and sulfanilamide in the cases of two strains of *Leuconostoc* one of which, *L. dextranicum* 8086, requires folic acid. As is seen from the table, growth of the other strain, *L. mesentcroides* 535, is stimulated by the addition of folic acid, although the polysaccharide yield is

TABLE 1

Effect of added PAB and sulfanilamide on dextran yield and acid production in cp sucrose casein hydrolyzate medium

ADDED PAB	ADDED SULFANILAMIDE	TIME	ACID PRODUUCTION*		DEXTRAN YIELD	
			L mes. 535	L. dex. elai	L. mes. 535	L. dex. elai
µg/ml	µg/ml	hr	ml	ml	%	%
0	0	48	11.5	7.1	43	48
1,000	0	48	9.3	4.8	47	32
500	0	48	10.0	6.7	46	39
100	0	48	10.8	6.7	45	37
1	0	48	11.9	7.0	45	42
0	2,000	48	1.1	0.4	†	†
		72	1.4	0.9	25	18
0	1,000	48	1.3	0.6	†	†
		72	1.9	1.1	39	15
0	100	48	1.6	1.1	†	12
		72	2.0	2.3	38	40

The basal medium contained no added PAB or folic acid.

* In this and subsequent tables, as ml 0.01 N NaOH used per ml medium.

† Less than 5 per cent.

approximately equivalent with or without added vitamin. Sulfanilamide in a relatively low concentration inhibited acid production and slowed dextran synthesis without affecting ultimate polysaccharide yield. In the presence of added folic acid both organisms were insensitive to the effects of high levels of the sulfonamide, as has been noted for other lactic acid organisms (Lampen and Jones, 1946). It was thought that if *p*-aminobenzoic acid functions directly in dextran synthesis, rather than as a precursor of folic acid, it might be possible to block this action by high concentrations of sulfanilamide in the presence of added folic acid. The failure to influence polysaccharide yield by this means again suggests that *p*-aminobenzoic acid is not involved in dextran synthesis, at least, by these strains.

The data so far presented were obtained with strains of *Leuconostoc* usually giving only low or moderate yields of dextran even after prolonged incubation. In table 3 are recorded data on the effect of folic acid, *p*-aminobenzoic acid, and

TABLE 2

Effect of added folic acid and sulfanilamide on dextran yield and acid production in cp sucrose casein hydrolyzate medium

FOLIC ACID	SULFANILAMIDE	TIME	ACID PRODUCTION		DEXTRAN YIELD	
			L. mes. 535	L. dex. 8086	L. mes. 535	L. dex. 8086
µg/ml	µg/ml	hr	ml	ml	%	%
0	0	24	0.5	0.1	*	*
		48	8.7	0.4	59	*
0.001	0	24	4.1	2.1	28	13
		48	11.1	10.5	60	36
0.01	0	24	5.8	4.6	55	14
		48	11.1	11.8	57	36
0	10	48	2.1	0.1	22	*
		72	3.3	0.4	55	*
0.01	1,000	24	4.7	2.2	52	*
		48	11.5	11.7	58	30
0.01	100	24	4.8	2.7	49	12
		48	11.2	12.5	—	34

* Less than 5 per cent.

TABLE 3

Effect of folic acid, PAB, and raw sugar on dextran yield and acid production in casein hydrolyzate medium by L. dextranicum elai

SUCROSE	ADDED FOLIC ACID	ADDED PAB	TIME	ACID PRODUCTION	DEXTRAN YIELD
	µg/ml	µg/ml	hr	ml	%
cp	0	0	24	0.1	*
			48	5.1	54
			72	9.5	100
cp	0.001	0	24	0.1	*
			48	5.1	69
			72	9.5	100
cp	0.01	0	24	0.1	*
			48	6.0	78
			72	10.5	100
cp	0	0.001	24	0.1	*
			48	4.5	59
			72	9.7	100
cp	0	0.01	24	0.1	*
			48	4.6	55
			72	10.2	100
Raw	0	0	24	4.4	25
			48	13.5	100
			72	14.7	
Raw†	0	0	24	0.6	18
			48	9.3	97
			72	11.6	100

* Less than 5 per cent.
† Adsorbed at pH 3 with norit A.

raw sugar on acid and dextran production by a strain, L. *dextranicum* elai, that consistently gives yields of dextran approaching theoretical conversion of the glucose half of the sucrose molecule into the polysaccharide. This strain does not require the addition of folic acid for growth, although it was found to be stimulated by the vitamin. Added p-aminobenzoic acid did not affect the rate of dextran synthesis. In contrast, substitution of raw cane sugar for cp sucrose resulted in marked stimulation of both acid and dextran production. When the raw sugar was treated in aqueous solution with norit A at pH 3 for 30 minutes prior to being added to the medium, the rates of acid and dextran synthesis decreased, although they still exceeded those obtained with added folic acid or p-aminobenzoic acid. The fractionation of raw sugar by this and other methods will be reported at a later date.

TABLE 4

Effect of folic acid, PAB, and raw sugar on dextran yield and acid production in casein hydrolyzate medium by L. mesenteroides 683

SUCROSE	ADDED FOLIC ACID	ADDED PAB	TIME	ACID PRODUCTION	DEXTRAN YIELD
	µg/ml	µg/ml	hr	ml	%
cp	0	0	24	0.1	*
			48	0.1	*
cp	0	0.001	24	0.1	*
			48	0.1	*
cp	0.01	0	24	5.8	63
			48	11.8	76
cp	0.01	1,000	24	6.4	45
			48	11.0	60
cp	0.01	0.001	24	7.2	62
			48	11.3	79
Raw	0	0	24	0.9	12
			48	1.0	26

* Less than 5 per cent.

Table 4 presents corresponding results in the case of L. *mesenteroides* 683, a strain that requires folic acid. p-Aminobenzoic acid was supplied in a range covering a 10^6 variation in concentration, the resulting effect on dextran synthesis being one of slight inhibition at the higher levels. Substitution of raw cane sugar for cp sucrose in the absence of added vitamins permitted only a low level of growth and dextran formation. This result indicates that raw sugar is a poor source of folic acid and suggests, when taken in conjunction with the data in table 3, that the stimulating effect of raw sugar is not due to its content of this vitamin.

The results so far discussed were obtained in casein hydrolyzate media containing, in addition to various salts and vitamins, the purines, adenine and xanthine. Such media satisfactorily supported growth and dextran formation with all the strains of *Leuconostoc* utilized, the substitution of raw cane sugar for cp sucrose generally resulting in increased rates of acid and dextran production. A chemically defined medium containing 19 amino acids, vitamins, salts,

adenine, xanthine, guanine, uracil, and thymine in the concentrations described in the preceding paper was found incapable of supporting the growth of all the various strains.

In table 5 are presented data for the effects of folic acid, p-aminobenzoic acid, and sulfanilamide on a strain, L. mesenteroides 683, which grew in this chemically defined medium. Growth and dextran synthesis in the absence of folic acid, though better than that observed in casein hydrolyzate, still were low. Thus, in contrast to results reported for some other lactic acid bacteria (Stokes, 1944), thymine apparently has only a limited ability to replace the folic acid requirement of this organism. The addition of folic acid in concentrations of 0.001 and 0.01 µg per ml strongly stimulated acid production and polysaccharide synthesis, titratable acidity being favored by the higher level of the vitamin and dextran formation by the lower. Supplementation of the medium with both folic acid and p-aminobenzoic acid gave results similar to those noted in casein hydrolyzate,

TABLE 5

Effect of folic acid, PAB, and sulfanilamide on dextran yield and acid production by L. mesenteroides 683 in a synthetic medium

(48 hours' incubation time)

SUCROSE	ADDED FOLIC ACID	ADDED PAB	ADDED SULFANILAMIDE	ACID PRODUCTION	DEXTRAN YIELD
	µg/ml	µg/ml	µg/ml	ml	%
cp	0	0	0	1.3	19
cp	0.001	0	0	8.6	67
cp	0.01	0	0	12.0	58
cp	0.01	0.001	0	11.9	59
cp	0.01	100	0	10.6	54
cp	0.01	0	1,000	7.9	65

i.e., inhibition at a high level of p-aminobenzoic acid. The effect on L. mesenteroides 683 of a high level of sulfanilamide in the presence of folic acid was similar to that previously recorded for strain 535 in table 3. The failure to decrease dextran synthesis by this means again indicates the noninvolvement of p-aminobenzoic acid in polysaccharide formation by these organisms.

To investigate further the effect of raw sugar in various media, acid production and dextran synthesis by three organisms were compared in a chemically defined medium and in one containing Difco peptone, as summarized in table 6. When added to the complete chemically defined medium, raw sugar caused a relatively greater stimulation in titratable acidity than in dextran synthesis. In the cp sucrose peptone (Difco) medium without added vitamins, the growth of all strains was retarded. In contrast, moderate growth with high yields of dextran was observed with two of the organisms when raw sugar was substituted for the cp sucrose. Results of this kind probably have been the basis of the reported stimulation of dextran synthesis by materials such as raw sugar (Carruthers and Cooper, 1936; Stacey and Youd, 1938). From table 6 it is seen that the differ-

ence between cp sucrose and raw sugar can be partially eliminated simply by supplying a complete mixture of vitamins. There is, nevertheless, a very definite stimulation by raw sugar which cannot be duplicated by the vitamin mixture and which is especially manifested in enhancement of growth and polysaccharide synthesis early in the incubation period, as shown, for example, in table 3. Also to be noted in table 6 is the tendency, with high dextran-forming strains such as *L. mesenteroides* 683, for an inverse relationship to exist between titratable acidity and polysaccharide yield.

TABLE 6

Effect of added vitamins and raw sugar on dextran yields and acid production in synthetic and peptone media

(48 hours' incubation)

SUCROSE	ADDED VITAMINS	ACID PRODUCTION			DEXTRAN YIELD		
		L. mes. 683	*L. mes.* 535	*L. dex.* 8086	*L. mes.* 683	*L. mes.* 535	*L. dex.* 8086
Amino acid, purine, pyrimidine medium*							
		ml	*ml*	*ml*	%	%	%
cp	9†	11.9	4.2	4.8	59	43	16
Raw	9†	14.6	7.8	8.6	64	56	21
Difco peptone medium‡							
cp	0	0.9	1.7	1.0	10	20	§
Raw	0	4.1	2.9	1.7	83	58	19
cp	9†	9.3	5.8	6.4	73	56	26
Raw	9†	11.9	7.1	8.0	73	62	36

* Nineteen amino acids, adenine, guanine, xanthine, thymine, and uracil.
† Folic acid, PAB, pyridoxal, pyridoxamine, riboflavin, thiamine, pantothenic acid, nicotinic acid, and biotin.
‡ 0.5 per cent concentration.
§ Less than 5 per cent.

Recently Stacey and Swift (1948) described dextran synthesis by *Leuconostoc* in a medium containing inorganic salts and a low concentration of peptone with *p*-aminobenzoic acid (500 μg per ml) as the only added vitamin. In similar vitamin-deficient media we have observed occasional stimulation of growth and dextran synthesis by the addition of *p*-aminobenzoic acid up to the level of 100 μg per ml. However, in view of the results obtained in more complete media, it is believed that this is a nonspecific stimulation not related to the possible presence of this vitamin in raw sugar.

SUMMARY

Low levels of *p*-aminobenzoic acid failed to stimulate dextran synthesis, and higher concentrations retarded polysaccharide formation by the strains of *Leuconostoc* employed. In the case of folic-acid-independent strains, sulfanilamide produced a greater inhibition of acid production than of dextran synthesis. In the presence of added folic acid, sulfanilamide in high concentrations did not affect polysaccharide formation by *Leuconostoc* strains that were dependent on this vitamin. These results indicate that *p*-aminobenzoic acid is not involved in dextran synthesis by the strains of *Leuconostoc* studied. The stimulating effect of raw cane sugar on growth and dextran synthesis could not be duplicated by *p*-aminobenzoic acid, folic acid, or a mixture of nine B vitamins.

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THE UPTAKE OF C¹⁴ OF CARBOXYL-LABELED GLYCINE INTO THE PROTEIN OF TORULA UTILIS[1]

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Protein synthesis in living tissues has been studied recently by several investigators. The uptake by liver slices of radio-sulfur administered as methionine and labeled with S^{35} was demonstrated by Melchoir and Tarver (1947). That the process was aerobic was shown by Franz, Loftfield, and Miller (1947), and that the azide ion was inhibitory was shown by Winnick, Friedberg, and Greenberg (1947). The desirability of using microorganisms in studies of this sort was suggested by Melchoir, Mellody, and Klotz (1948), who demonstrated that

TABLE 1

Influence of various factors on uptake of C^{14} from glycine by Torula utilis var. major

TREATMENT	RELATIVE RADIOACTIVITY OF PROTEIN
No change (standard medium*).....	100
Glucose omitted from standard medium.	29
CaCl₂ omitted..	63
MgSO₄ omitted	89
O₂ atmosphere replaced by N₂ . ..	57
0.01 M Cyanide added..	8
Drying and subsequent grinding of yeast	0

* The standard medium contained per liter: glucose, 50 g; $(NH_4)H_2PO_4$, 3 g; $(NH_4)_2HPO_4$, 4 g; K_2SO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 1 g; and $CaCl_2$, 0.5 g.

Escherichia coli cells incorporated S^{35}-labeled methionine into cell protein by an enzymatic process without some of the undesirable side reactions observed with mammalian tissue slices.

Roine (1947) employed *Torula utilis* in studies of protein synthesis, pointing out that multiplication was rapid and protein easily elaborated from inorganic nitrogen without the addition of undefined "growth substances."

In studies reported here, cells of *Torula utilis* var. *major* were incubated with carboxyl-labeled glycine at 29 C in an atmosphere of oxygen. The uptake of C^{14} and the influence of various factors on its incorporation in cell protein are shown in table 1.

Cells were incubated 45 minutes in flasks containing 2 ml medium and a trace of C^{14}-labeled glycine, which had an activity of 1,360 counts per second. The cell suspensions in each flask were equivalent to 30 mg of protein. The cells were

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washed three times with their respective media before incubation with labeled amino acid.

At the end of 45 minutes of incubation with labeled glycine, the protein of the cell suspensions was precipitated with 5 per cent trichloroacetic acid. The precipitates were centrifuged and washed four times with 5 per cent trichloroacetic acid containing inert glycine and then with hot 95 per cent ethylalcohol. The protein was then suspended in hot alcohol-ether mixture (3:1), collected on filter paper, weighed, and counted.

It will be seen from table 1 that the incorporation of C^{14} into cell protein was inhibited by cyanide and by anaerobic conditions, and was enhanced by glucose and calcium. Drying and grinding of the yeast cells prevented C^{14} uptake. The activity of protein from cells incubated in oxygen with radio-glycine average 0.66 counts per second per milligram of protein from materials prepared on different days.

It would appear that an oxidative mechanism is necessary for C^{14} uptake in microorganisms as well as in mammalian tissues. That this mechanism is enzymatic is further indicated by its inhibition by cyanide and by the fact that dried cells are without synthetic activity.

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THE ELECTRON MICROSCOPY OF HEATED BACTERIA

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In several recent studies it has been shown that bacteria stained by the Feulgen reaction and the Robinow technique exhibit an internal structure visible under the optical microscope (for full references to the literature see Malmgren and Hedén, 1947; Knaysi, 1944; Dubos, 1945). By some this has been interpreted as evidence for the existence of a definite bacterial nucleus (see Bisset, 1948). It is important in this connection to ascertain what structures are visible, at the higher resolutions of the electron microscope, in such stained bacteria and in bacteria digested with nucleases. Both the Feulgen staining and the Robinow technique as ordinarily used involve hydrolysis in HCl and heating at 60 C. This paper describes control experiments to test the effects of heating to this temperature on the structure to be seen in bacterial protoplasm.

METHODS

In all experiments the test organisms were from cultures of the B strain of *Escherichia coli*. They were grown by inoculating an Erlenmeyer flask containing 200 ml of Difco nutrient broth with enough of an 18-hour agar slant culture to give a density of about 10^8 organisms per milliliter. The inoculated flask was rocked in a water bath at 37 C for 1 hour, when observation in a phase contrast microscope showed that the bacteria exhibited the appearance characteristic of the beginning of the logarithmic stage of growth. In this condition the average bacterial volume is at a maximum. At the end of this hour of incubation the broth culture was centrifuged and the sediment resuspended in physiological saline. Washing was accomplished by repeating the sedimentation and resuspension in saline. After a third sedimentation, the bacteria were finally suspended in a comparatively large volume of the medium in which they were to undergo heating. Small volumes of this purified suspension (0.2 ml in small centrifuge tubes to ensure quick thermal equilibrium with the bath) were heated by immersion for 10 minutes in a water bath of carefully controlled temperature. This period of time was chosen because it corresponds to that conventionally used in the Feulgen test. After being heated, the suspension was centrifuged and the sediment resuspended in distilled water. For electron microscopy, microdrops of this aqueous suspension were placed on the usual collodion-covered grid and, when dry, shadowed with a mixture of gold and manganin wire in equal amounts.

RESULTS

Typical unheated bacteria are shown in figures 1 to 3. Those of the first figure are taken from the 18-hour slant before incubation; those of figures 2 and 3 are
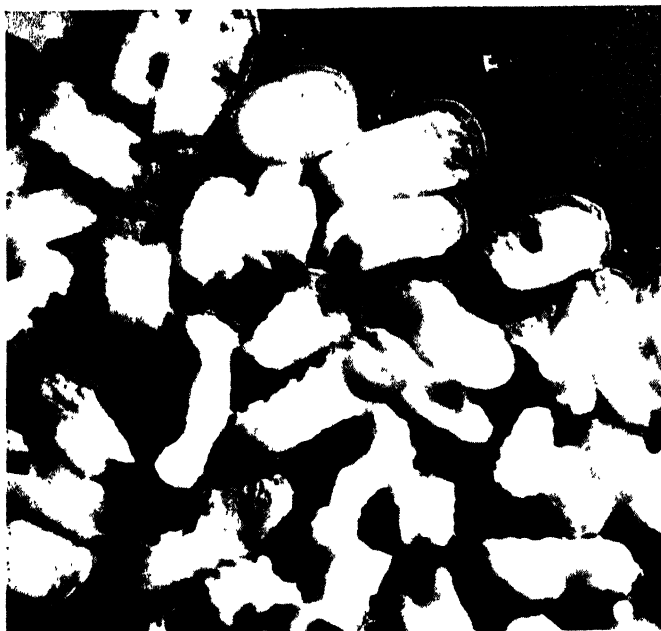
Figure 1. An electron micrograph of bacteria from an 18-hour slant of *E. coli*. Magnification 16,000 ×.
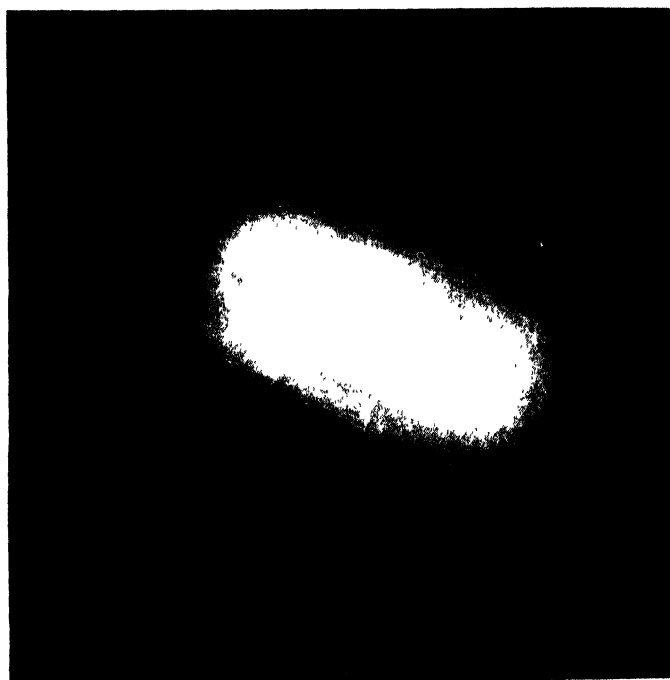

Figure 2. A typical colon bacillus from a 1-hour broth culture. Magnification 28,000 ×.
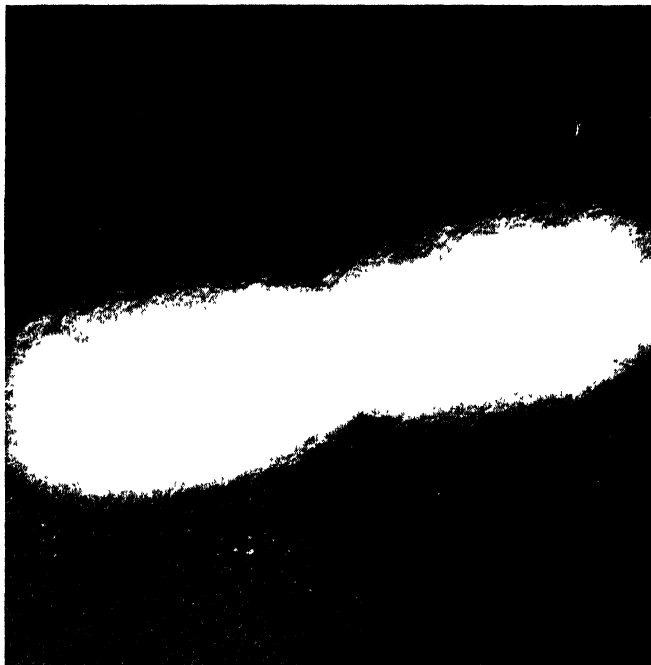
Figure 3 A pair of dividing colon bacilli from a 1-hour broth culture of *E. coli*. Magnification 28,000 ×.

Figure 4. Several colon bacilli prepared as described in the text after being heated for 10 minutes in distilled water at 60 C. Magnification 16,000 ×.

Figure 5. A colon bacillus after being heated for 10 minutes in water at 50 C. Magnification 16,000 ×.

Figure 6. Colón bacilli after being heated for 10 minutes in physiological saline at 40 C. Magnification 16,000 ×.

from a 1-hour broth culture. The greatly increased size of these young organisms and the uniformity of their protoplasmic contents are very apparent.

Figure 7. Typical colon bacilli after being heated for 10 minutes in saline at 50 C. Magnification 16,000 ×.
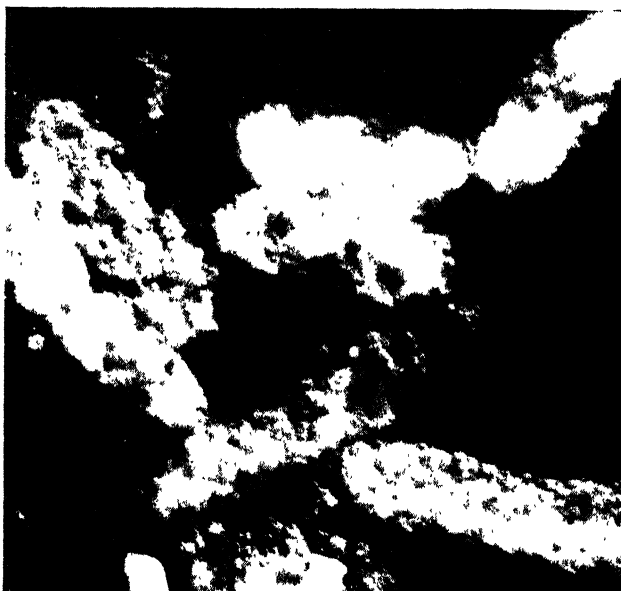
Figure 8. Colon bacilli after being heated for 10 minutes in saline at 53 C. Magnification 16,000 ×.

In all, four sets of experiments were made. In the first set heating was accomplished in distilled water suspension, in two other sets the heating was in physi-
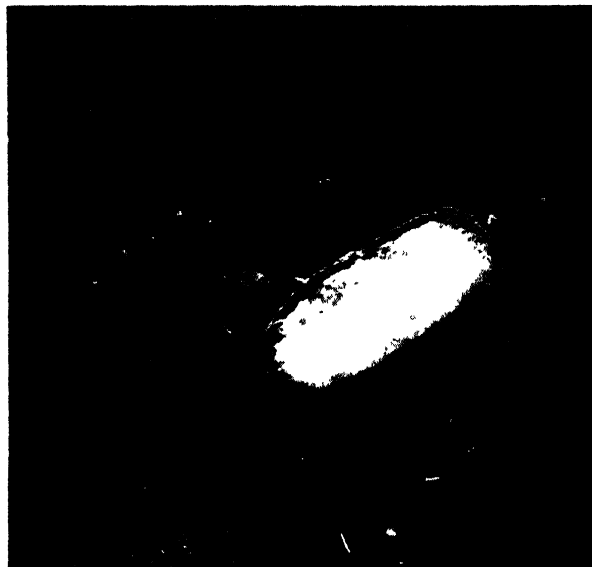
Figure 9. Colon bacilli after being heated for 10 minutes in saline at 55 C. Magnification 18,000 ×.

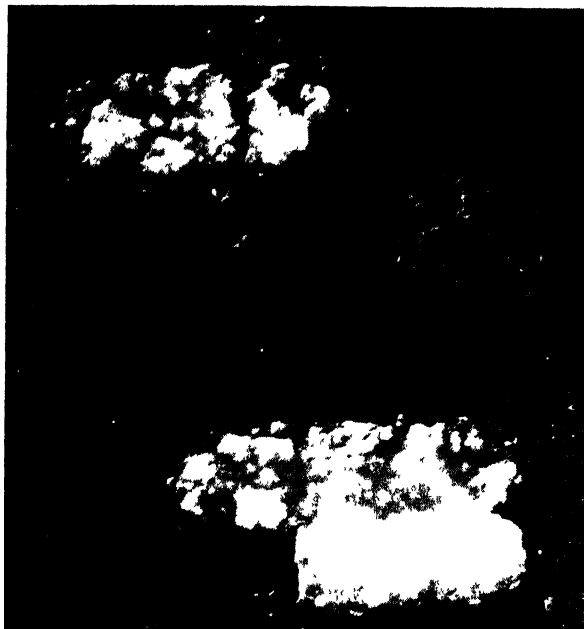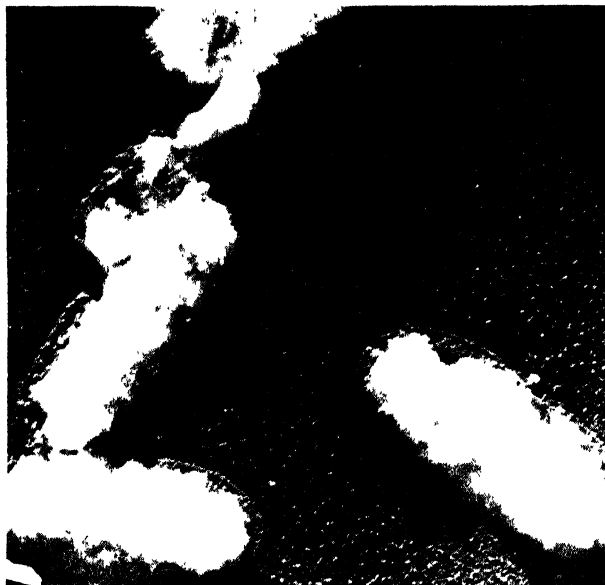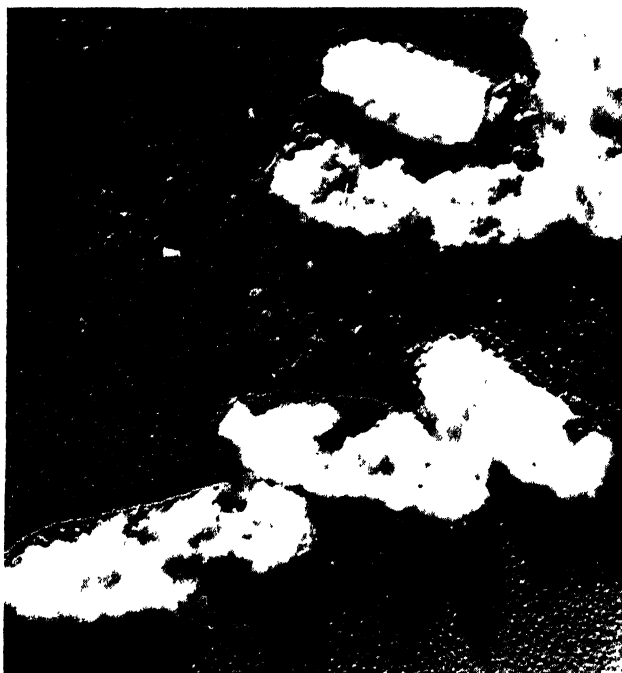*Figure 10.* Colon bacilli after being heated for 10 minutes in saline at 55 C. Magnification 16,000 ×.

Figure 11. Colon bacilli heated for 10 minutes in saline at 45 C and then kept in cold water overnight as described in the text. Magnification 18,000 ×

Figure 12. Colon bacilli heated for 10 minutes in saline at 55 C and then stored overnight in cold water. Magnification 18,000 ×.

ological saline, and in the fourth a borate buffer of pH 7.6 was employed in place of the saline. After being heated in distilled water at 60 C, the cellular protoplasm (figure 4) developed a coarsely granular structure. As figure 5 indicates, granulation began at 50 C; it was quite complete after 10 minutes' heating at 55 C.

This coagulation takes place at a lower temperature if the heating occurs in the presence of saline. As can be seen from figure 6, bacteria exposed to only 40 C have lost some of the protoplasmic homogeneity of unheated young organisms (figures 2 and 3). Heating at 50 C yields a complete granulation (figure 7) that, as figures 8 to 10 demonstrate, is not to be distinguished from that produced at higher temperatures.

In view of this rather surprising sensitivity of the bacteria to heating in the presence of saline, a third set of experiments was performed to ascertain whether this protoplasmic coagulation could be reversed by letting the heated bacteria stand for some time in the presence of water. The bacteria, heated in saline, sedimented, and resuspended in distilled water, were left to stand overnight in the water, at icebox temperature, before being placed on grids for examination. The protoplasm of cells heated below 50 C became homogeneous again under this treatment. As can be seen from figure 11, there is a partial reversibility of texture in bacteria heated at 45 C, but heating above 50 C (figure 12) produces a change that is not reversible.

The experiments in the last group, in borate buffer, were carried out as a control on digestions with ribonuclease. They show that the changes taking place below 50 C are small, but that above this temperature they are like those seen in the other experiments.

These results demonstrate beyond question that heating colon bacilli at 60 C granulates their protoplasm in an irreversible fashion. Since such heating is an essential step in the Feulgen staining of this and other protoplasm and is commonly used to facilitate digestion with nucleases, this fact must be given careful consideration when conclusions are being drawn from the appearance of heated protoplasm concerning the distribution of its nucleic acids.

SUMMARY

The protoplasm of young colon bacilli becomes coarsely granular when heated for 10 minutes in water at 55 C or above. When the organisms are heated in saline, the beginnings of such a granulation become evident at as low a temperature as 40 C; it is complete above 50 C. Below, but not above, this temperature the change in texture can be reversed by allowing the bacteria to stand overnight suspended in distilled water.

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SURVIVOR CURVES OF BACTERIA EXPOSED TO SURFACE-ACTIVE AGENTS[1]

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Reports on bacterial death studies since the basic work of Krönig and Paul (1896) have generally recognized that the death process of bacteria exposed to unfavorable environmental conditions follows a uniform and consistent course. The constancy of the rate of death is the outstanding feature; the term, "constant death rate," means that the number of bacteria that die per time unit is a constant percentage of the number of living organisms at the beginning of this time unit (Rahn, 1945). The order of death of organisms may be determined by computation of the value of the death rate constant, K, in the formula:

$$K = \frac{\log b - \log B}{t}$$

where b equals the number of organisms at the beginning of the time unit; B equals the number of organisms at the end of the time unit; and t equals the time unit, i.e., the period of exposure. If the order of death is logarithmic, as has generally been conceded to be true in bacterial death, the value of K remains relatively constant throughout the exposure period. Although differences in opinion as to the interpretation to be attached to the form of the survivor curve exist, most workers have assumed that the phenomenon was due to fundamental chemical and physical factors involving some basic process analogous to a monomolecular reaction (Madsen and Nyman, 1907; Chick, 1908; Lee and Gilbert, 1918; Watkins and Winslow, 1932; Rahn, 1945).

In the course of studies on the bactericidal properties of the quaternary ammonium compounds, the observation of discordant results with phenol coefficient methods led the authors to employ a plate count, survivor curve method of germicidal evaluation. Aberrant disinfection velocity curves were reported by one of the authors (McCulloch, 1947) for various quaternary ammonium compounds. The theory was advanced that these cationic agents tend to promote clumping or agglomeration of the exposed test organisms, reducing the plate count in earlier minutes of exposure without necessarily reducing the number of viable organisms. The clumping of treated organisms, with their adherence to glass surfaces, had been observed experimentally (McCulloch and Migaki, 1947).

The following studies were conducted to investigate some of the factors other than a clumping phenomenon which might appreciably influence the death rate

[2] Died, December 1, 1948.

of organisms exposed to quaternary ammonium compounds and be responsible for the observed atypical type of survivor curve. Factors considered for this report were the possible presence of unusual variations in resistance among individual cells exposed, the effect of the age of exposed cells on their relative resistance, and the possible exhaustion of active free detergent in the exposure test suspension.

EXPERIMENTAL DATA

Determination of Survivor Curve Patterns of Organisms Exposed to Cationic, Anionic, and Nonionic Surface-active Agents

Materials. Laboratory cultures of *Micrococcus pyogenes* var. *aureus, Escherichia coli, Pseudomonas aeruginosa,* and spores of *Bacillus subtilis* were used. The representative detergents selected were classified in three categories: cationic— "hyamine 1622," "roccal," "emulsept," "teramine"; anionic—"dreft," "tide," "santomerse 3," "syntex A," "oronite D-40"; nonionic—"S.T. 37," "triton 100," "triton X30." The Food and Drug Administration broth medium (Reuhle and Brewer) was used to culture the test organisms. The medium used for plating was phenol red agar base (Difco) to which had been added 1.0 per cent glucose. In experiments involving either *P. aeruginosa* or *B. subtilis,* a film of 2.0 per cent agar was poured over the solidified medium in the plates to prevent surface growth of spreaders. Standard 99-ml milk dilution bottles containing sterile distilled water were used routinely. The first dilutions of a bacteria-detergent mixture were always made in blanks containing 10 per cent sterile evaporated milk, since it was found that this concentration of milk was able to provide extensive surface for competitive absorption and to neutralize the action of the surface-active agent against the organisms.

Test procedure. (1) An approximately 24-hour culture of the test organism, which had been transferred on 15 consecutive days prior to the performance of the tests, was shaken thoroughly and filtered through cotton. (2) Fresh stock solutions of surface-active agents were prepared in sterile water in volumetric flasks. (3) Ten ml of filtered culture were added to 100 ml of surface-active agent solution held in a constant temperature water bath. The mixture was immediately shaken and agitated 25 times and replaced in the water bath for the exposure period. (4) Samples of a bacteria-detergent mixture, usually of 1.0 ml, were withdrawn at given time intervals for dilution and plating. Triplicate plates were generally poured for each dilution. (5) The plates were incubated at 37 C and read after 24 hours and 48 hours. The number of survivors was determined by counting plates showing well-distributed colonies in the range of 30 to 300, taking the average, and multiplying it by the appropriate dilution factor. (6) Controls using 10 ml of bacterial culture and 100 ml of sterile distilled water were run simultaneously with all tests to establish the initial number of organisms employed in each experiment and to determine possible germicidal action of suspending waters or temperatures.

Results. The data presented in table 1 and figures 1 and 2 are typical of the results obtained using all of the agents mentioned against the given test cultures.

TABLE 1

Micrococcus pyogenes aureus exposed to 1:100,000 hyamine at 30 C

TIME	PLATE COUNT NUMBERS	LOGARITHMS OF PLATE COUNTS	K*
Before exposure:........................	39,000,000	7.5911	
After exposure:			
1 min................................	2,520,000	6.4014	1.1897
2 min................................	660,000	5.8195	0.5819
5 min................................	320,000	5.5051	0.1048
10 min................................	125,000	5.0969	0.0816
20 min................................	36,500	4.5823	0.0535
30 min................................	12,900	4.1108	0.0451
60 min................................	5,620	3.7497	0.0120
120 min................................	1,570	3.1959	0.0092

$$* K = \frac{1}{t} \log \frac{\text{Initial number of organisms exposed}}{\text{Number of survivors after exposure, } (t)}.$$
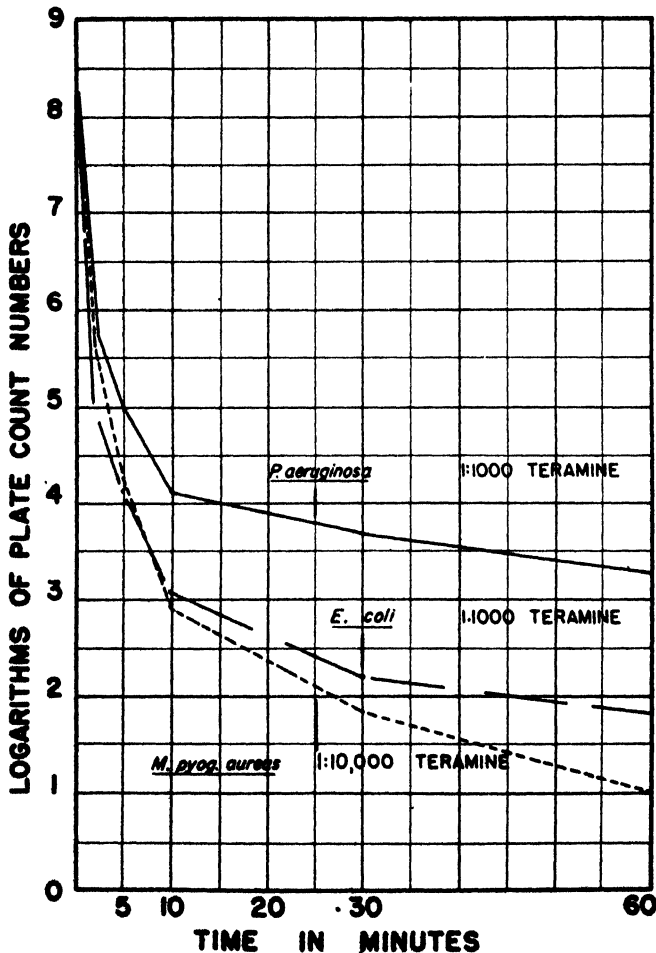

Figure 1. Survivor curves of *Escherichia coli, Micrococcus pyogenes* var. *aureus,* and *Pseudomonas aeruginosa* exposed to teramine at 21 C.

It is of particular interest to note that the value of the disinfection velocity constant was far from constant in any of the trials. When the same procedure was applied and phenol was used as the disinfectant against the test organisms, the survivor curves obtained were of a fairly constant, straight-line type. The data in table 1 showed the rate of bacterial reduction to be extremely rapid during the first 2 minutes, followed by a slower rate of kill which continued until the end of the tenth minute. Between the tenth and twentieth minutes the rate of kill again declined appreciably, which fact might indicate that the bacterial population was comprised of components of different degrees of resistance.

The determination of the survivor curve pattern presented by resistance spores of *Bacillus subtilis* exposed to cationic and anionic detergents revealed results of interest and possible significance. A laboratory strain of *Bacillus subtilis* was cultured on the surface of nutrient agar in a Blake bottle and allowed to incubate for a week. The growth was harvested with sterile water and shaken vigorously,
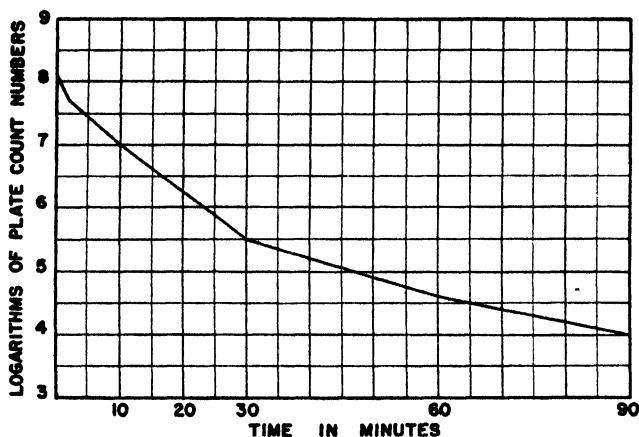
Figure 2. Survivor curve of *Micrococcus pyogenes* var. *aureus* exposed to 1:10 S.T. 37 at 21 C.

and the vegetative cells were destroyed by being heated at 60 C for 30 minutes. The suspension was then poured through sterile, fine, dry silica sand; the sand was desiccated and the material stored in sterile bottles at 4 to 8 C. The spore count of this sand material was found to remain constant during the months the trials were conducted, and there was no change in the phenol resistance of the spores. Shaken with 100 ml of sterile water, 1.8 grams of the sand gave a count of 20 to 30,000,000 spores per ml. Ten ml of such a spore suspension were added to 100 ml of detergent solution in the tests.

The results are shown in figure 3. It is interesting to note the marked initial reduction in plate count of the exposed spores followed by negligible reduction. It is highly improbable that this observed initial reduction actually represented death of the spores to the extent indicated by the plate count numbers.

The Possible Presence of Unusual Variations in Resistance among Individual Cells

Past observations of abrupt breaks in survivor curve patterns of bacteria have been attributed to the presence of unusual variations in resistance of the

test organisms or to the effect of the age of cells upon relative resistance. Unusually resistant variants appearing at some stage of development of the culture could produce a decreasing value of K and a survivor curve such as is shown in this report. Experiments were performed to determine whether subcultures of the survivors of different exposure periods differed appreciably in resistance from one another and from the original culture.

Test procedure. Colonies on the survivor plates of exposure tests were picked off and inoculated into tubes of F.D.A. broth. Approximately 24-hour cultures of these substrains were compared with the original culture for resistance to the surface-active agents. The procedure and conditions of the original tests were duplicated.

In a second series of experiments, cultures of *Escherichia coli* were exposed to sufficient quaternary ammonium compound to reduce the viable colony count by at least one million times. The survivors were inoculated into tubes of F.D.A. broth and, following incubation, were also exposed to a given concentration of
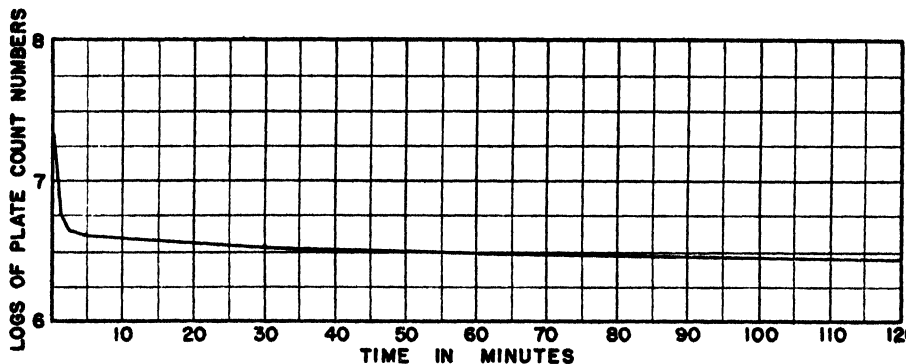
Figure 3. Survivor curve of *Bacillus subtilis* spores exposed to 1:100 hyamine 1622 at 21 C.

quaternary ammonium compound. Exposure tests were repeated on these cultures on 6 successive days.

Results. The results obtained in the series gave no indication that the survivors of such exposures were more or less resistant to the surface-active agents than their parent strains. The substrains displayed the same type of survivor curve and similar degrees of resistance.

The Possible Effect of the Age of Cells on Relative Resistance

As previously mentioned, the age of the cells exposed to a disinfecting agent has been suggested as one factor responsible for a nonlogarithmic order of death. It is generally accepted that young cells are more sensitive to unfavorable environments than are older cells. Were the survivors curves obtained in this study due to the presence in the test culture of a small number of old resistant cells that had perhaps remained in a resting stage when transferred to a new medium, it might follow that a young culture, transferred successfully for several times at short intervals, would eliminate such old cells. The survivor curve obtained using such a culture would follow the usual constant trend.

Test procedure. A culture of *Escherichia coli* was successively transferred for 5 times at 4-hour intervals. With the procedure outlined above, the final sub-culture was tested at various ages against a 1:30,000 hyamine 1622 solution.

Results. The results are presented in figure 4. Although the resistance of the young cultures is less than that of the older cultures, the trends are similar, and
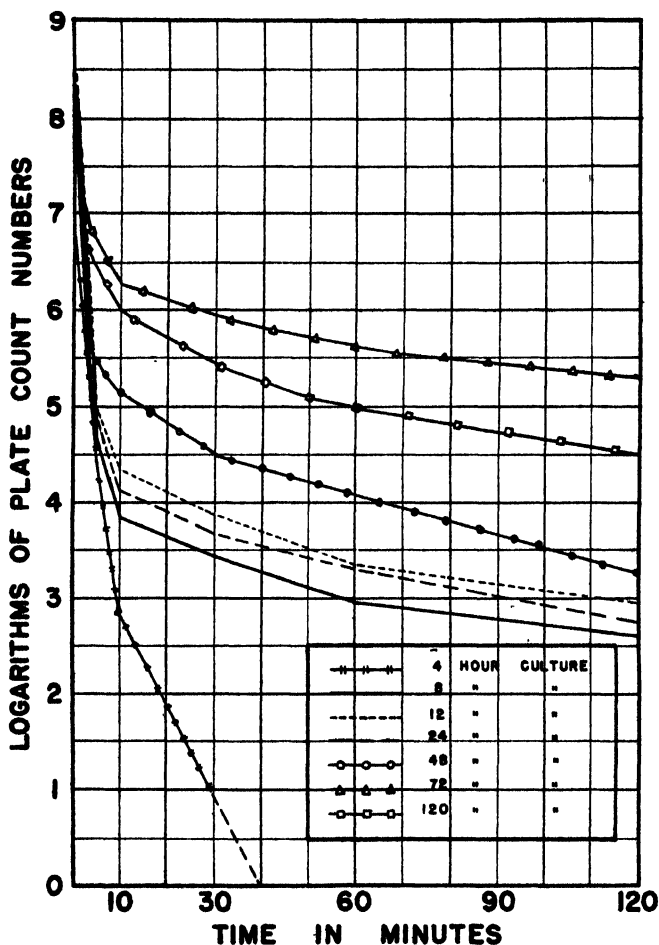
Figure 4. Survivor curves of cultures of *Escherichia coli* of different ages exposed to 1:30,000 hyamine 1622 at 21 C.

in all instances they depart from the straight-line graph. It does not seem reasonable to attribute the phenomenon of the atypical survivor curve presented by organisms exposed to surface-active agents to the effect of age upon the relative resistance of the cells.

The Possible Exhaustion of Active Detergent in the Test Suspension

Another explanation proposed for the asymptotic curves was that of an exhaustion of the active detergent molecules in a bacteria-detergent mixture. The

effectively destructive concentration of the surface-active agent may have become diminished so rapidly during the first few minutes of the exposure period that, in later minutes of exposure, there was insufficient detergent present in the solution proper to reduce the bacterial population as rapidly or as efficiently. Molecules of surface-active agents, because of their very nature, tend to concentrate themselves at interfaces and on surfaces; the fact that cationic germicides are readily adsorbed on glass surfaces and can be readily removed from solution has been demonstrated (Miller *et al.*, 1943; Weber and Black, 1948).

Test procedure. To determine whether such a factor was responsible for the constantly decreasing death rate observed, the following procedure was devised: To 100 ml of detergent solution, 5 ml of a filtered 24-hour bacterial culture were added at each of 4 successive 20-minute intervals. The mixture was thoroughly
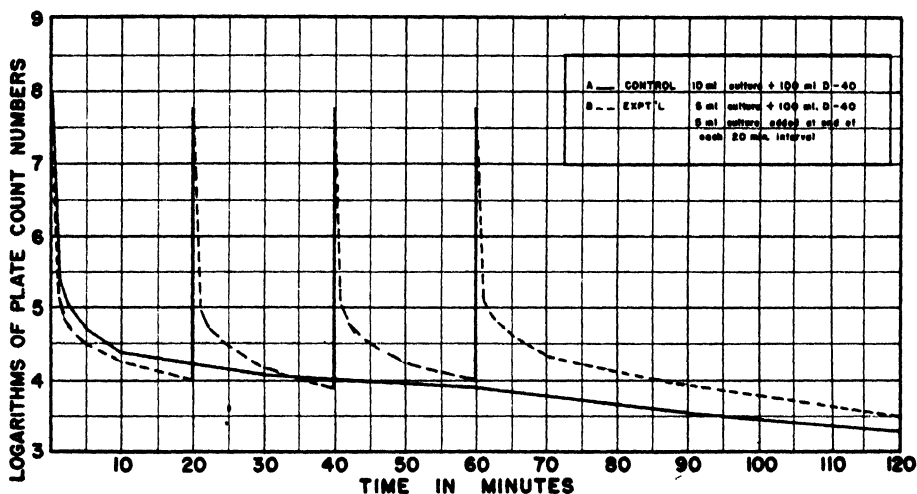
Figure 5. The effect on the survivor curve pattern of repeated additions of *Micrococcus pyogenes* var. *aureus* to 1:400 oronite D-40 at 44 C.

shaken after each addition of culture, and 1.0-ml samples were removed for dilution and plating at 1-, 2-, 5-, 10-, and 20-minute intervals following each culture addition. A control run using the standard proportions of 10 ml culture to 100 ml detergent was also made.

Results. Figure 5 shows the results obtained using oronite D-40 against *Micrococcus pyogenes* var. *aureus*. Following each culture addition, there was more than a 2.5 log drop in the survivor count at the end of the first minute of exposure. Actual exhaustion of the detergent is represented only by the very slight differences in the total plate count drop. The data seem to indicate conclusively that the concentration of free and active detergent in an exposure solution in which the proportions of bacterial culture to detergent solution are 1 to 10 is more than sufficient to exert its maximum action throughout the entire exposure period.

In a number of plate count studies it was noted that the counts of serial dilutions plated from a given sample of bacteria-detergent mixture were significantly

inconsistent, with the low dilution plates giving lower survivor counts per ml than the higher dilution plates. For example, a 10^{-3} plate would reveal 200 colonies or 200,000 survivors per ml, but a 10^{-4} plate would reveal 30 to 50 or 300,000 to 500,000 survivors per ml. To determine whether such discrepancies were due to error in the dilution technique itself, experiments were conducted substituting phenol and chlorine as the disinfectants. Plate counts of serial dilutions followed the normal pattern. Such results indicated that the process of dilution, which is necessarily accompanied by vigorous shaking and agitation, was reponsible for the release of additional viable cells.

DISCUSSION OF RESULTS

The results obtained in the foregoing experiments indicate that the observed atypical survivor curves of organisms exposed to surface-active agents cannot be adequately explained on the basis of (1) the presence or development of unusually resistant variants among the exposed organisms; (2) the effect of the age of cells upon relative resistance; (3) the exhaustion of active detergent in the exposure test suspension.

Evidence presented seems definitely to eliminate the latter two factors. The first of these factors cannot perhaps be conclusively discounted. However, the following observations do not substantiate the explanation that the cultures tested were comprised of growth phases or substrains possessing an unusual distribution of resistance against the surface-active agents: (1) The same survivor curve trend was observed in all trials with all types of cationic and anionic detergents and against all the bacterial cultures tested. (2) Parallel tests using phenol gave reasonably uniform values of the death rate constant, K, indicating a normal resistance distribution against phenol. (3) The same general pattern was observed with young and old cultures. (4) Organisms surviving exposure to the detergents did not give rise to substrains more resistant than the original culture.

The data presented seem to indicate that the observed survivor curves do not represent a true index of the death rate. The surface-active agents appear to effect an agglomeration of the exposed organisms. The observation that the process of dilution and the shaking accompanying it resulted in a release of greater numbers of viable cells and, consequently, in higher plate count readings is evidence in favor of this belief. Organisms exposed to cationic agents are known to undergo alteration of their surfaces and their surface electrical charge (Dyar and Ordal, 1946; Kivella et al., 1948). A rapid initial agglomeration, a clumping of exposed cells, or the formation of "bacteria-detergent complexes" could be anticipated as a result of such alteration.

With plate count methods, which give the number of colonies developing from a known volume of bacterial suspension, the colonies appearing on the plate represent the actual number of original bacteria only when such organisms are single units. If the cells are clumped together, one colony may easily represent a number of cells. In such a case, death would become evident only after the last of the cells within the clump had been killed; the plate counts would reveal the many viable cells within an agglomerate as a single unit, since the bound

cells would give rise to but a single colony. In the later intervals of the exposure period, however, when the rate of decrease appears to be extremely slow as determined by plate count readings, the actual destruction of organisms may be assumed to be taking place at a rate more rapid than was indicated. The survivor curves of bacterial cultures exposed to a surface-active agent seem, therefore, to represent not only chemical disinfection action, but also physical phenomena that are characteristic of these compounds.

SUMMARY

The factors involved in the production, using plate count methods, of the unusual survivor curves observed with bacterial populations exposed to various surface-active agents have been investigated. It is postulated that the survivor curves observed in the studies are a resultant, not only of direct chemical disinfection, but of certain physical effects exerted by these compounds and do not provide a true index to the rate of death.

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THE RELATIONSHIP BETWEEN GROWTH AND MUTATION IN PSEUDOMONAS FLUORESCENS

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Lwoff and Audureau (1941) have reported experiments which they interpret as evidence for the occurrence of spontaneous bacterial mutations[1] in the absence of growth. These workers discovered a positive biochemical mutation, involving the ability to use succinate as the source of energy, in the bacterium *Moraxella lwoffi*. *M. lwoffi* is an obligate aerobe with simple nutrient requirements and develops rapidly in a medium containing the necessary minerals, ammonia nitrogen, and any one of a variety of simple carbon compounds. The parent strain is unable to utilize succinate, but rare succinate-positive mutants are formed, being present to the extent of approximately one cell out of every 10^8 cells in parent clones. When small numbers of parent cells (approx. 10^5) are placed in tubes of mineral succinate medium, growth (as indicated by visible turbidity) becomes apparent after a variable length of time, ranging from 3 to 30 days. The growth in such positive cultures always consists of the succinate-positive mutant. The conclusions drawn by Lwoff and Audureau from such experiments were (1) that the parent strain cannot grow in a mineral succinate medium and, consequently, (2) that the variable time that elapses before visible turbidity appears is the time necessary for mutation to take place in a population of non-proliferating cells together with the time required for a mutant cell, once formed, to multiply up to the point of visible turbidity. Such an interpretation, if true, would necessitate a widening of Luria and Delbrück's (1943) definition of the mutation rate, which carries the definite implication that bacterial mutations occur only in the proliferating state. However, since the results obtained by Luria and Delbrück (1943) and Witkin (1947) have been interpreted as showing that, in the case of mutations to virus resistance, growth is necessary for mutations to occur, it appeared desirable to analyze somewhat more closely the type of situation met with in the experiments of Lwoff and Audureau before accepting their conclusions as valid. An opportunity to do so arose when a very similar positive biochemical mutant was discovered in an organism nutritionally similar to *Moraxella lwoffi*. The organism is *Pseudomonas fluorescens*, also an autotrophic aerobe (Lwoff *et al.*, 1946), and the mutation involves ability to utilize as a carbon source itaconate (methylene succinate), a substance not used by the parent strain.

MATERIALS AND METHODS

The organism used throughout this work was *Pseudomonas fluorescens*, strain A.3.7 (Stanier, 1948). The medium used for the growth of the organism and for

[1] By spontaneous mutations we mean mutations not deliberately induced by the investigators.

the determination of total viable counts consisted of yeast extract, 0.5 per cent; KH$_2$PO$_4$, 0.1 per cent; MgSO$_4$, 0.05 per cent; and agar, 1.5 per cent; the pH being adjusted to 7.2 (medium 1). For the isolation of the itaconate mutant and for the determination of the mutant count a synthetic medium of the following composition was used: sodium itaconate, 0.13 per cent; NH$_4$NO$_3$, 0.1 per cent; MgSO$_4$, 0.05 per cent; phosphate buffer, 0.1 per cent (pH 7.2); and agar, 1.5 per cent (medium 2). Except where otherwise indicated, a mixture of 10 per cent tap and 90 per cent distilled water was employed in preparing the media. A liquid itaconate medium (medium 3) was prepared as above with the omission of the agar.

Viable counts were performed by the method of surface streaking, using a rotating table. With a bent glass rod, 0.2 ml of a suitable dilution were spread evenly over the surface. In order to prevent the spreading of colonies, a piece of filter paper saturated with glycerol was placed on the lid of each dish prior to incubation. The surface streak method is just as reliable as ordinary plating (Snyder, 1947) and facilitates early observation in consequence of the more rapid growth of surface than of subsurface colonies.

<div align="center">RESULTS</div>

Discovery and isolation of the itaconate mutant. The mutant was discovered during an investigation of the organic compounds utilizable for growth by *Pseudomonas fluorescens.* When streaked on mineral agar plates containing sodium itaconate as the sole source of carbon, many strains give rise to very sparse growth, no greater than on a control plate devoid of added carbon source. If such plates are kept for several days, however, a few large colonies develop on the surface of the original growth. Clones isolated from such colonies by repeated streaking on itaconate agar possess the general morphological and physiological characters of the parent strain but differ from it by their ability to grow promptly and abundantly in media with itaconate as the sole carbon source.

The newly acquired property of these clones is extremely stable. Even after 30 consecutive daily subcultures in yeast extract without itaconate, the clones consist almost exclusively of itaconate-utilizing cells, as evidenced by the fact that the viable counts on yeast agar and on mineral itaconate agar show no significant differences.

The simplified fluctuation test. The random origin of the mutant was established by a series of experiments, analogous to those performed by Lwoff and Audureau (1941) with the succinate mutation in *Moraxella lwoffi.*

A suspension of parent cells from a 24-hour culture on yeast agar was washed twice with phosphate buffer by centrifugation, resuspended in phosphate buffer, and used to inoculate a flask of mineral itaconate medium (medium 3). After being mixed, this suspension was then distributed in 5-ml amounts into a series of sterile tubes, a viable count being made at the same time to determine the size of the inoculum per tube. The tubes were incubated at 30 C and examined daily for turbidity. The results of three such experiments are recorded in table 1.

An inoculum of a few mutant cells in medium 3 gives rise to visible turbidity after 48 hours at 30 C. Consequently, the appearance of visible turbidity within

2 days when an inoculum of parent cells is used indicated the probable introduction of at least one mutant cell with the inoculum. This has been taken into account in presenting the results in table 1, and the time of occurrence of mutation therein recorded has been calculated by subtracting 2 days from the time when visible turbidity was observed. In experiment 2 a mutant appears to have been present in 2 tubes out of 31 at the start of the experiment (visible turbidity after 48 hours). From this it can be calculated that the proportion of mutant to parent

TABLE 1

Simplified fluctuation tests as proof of the random origin of the itaconate mutant

TIME OF OCCURRENCE OF MUTATION, DAYS AFTER START OF EXPERIMENT*	NUMBER OF TUBES SHOWING MUTANTS IN EACH SUCCESSIVE PERIOD		
	Expt. 1	Expt. 2	Expt .3†
0	0	2	0
1	3	14	2
2	5	4	9
3	6	4	6
4	7	2	2
5	1	1	2
6	1	0	1
7	0	1	1
8	0	1	0
9	0	1	0
10	2	1	1
11–15	2		2
16–20	1		2
21–25	1		
26–27	1		
Initial viable count per tube..	1.8×10^6	2×10^7	1.4×10^6
Number of tubes used	30	31	30
Number of tubes with mutants at end of experiment..	30	31	28

* The time when visible turbidity was noted minus two days.
† Experiment terminated after 20 days.

in the inoculum was 1 to 3×10^8. This ratio is of the expected order in the light of the mutation rate as subsequently calculated.

The relationship between growth and mutation. Although the simplified fluctuation tests described above showed the mutational nature of itaconate utilization, they provided no information about the relationship between growth and mutation, and it was evident that only daily counts of the parent and mutant population would settle this question. Accordingly, an experiment identical in its preliminary phases to the ones described above was performed. Sixty tubes were used, three of which were immediately withdrawn for initial viable counts on the total and mutant population. The remainder were incubated at 30 C, and

each day thereafter three more not showing visible turbidity were withdrawn and similarly assayed. The viable mutant counts were made in order to ensure

TABLE 2

The relationship between growth and mutation

(Experiment no. 4)

DAYS AFTER START OF EXPERIMENT	TOTAL VIABLE COUNT PER TUBE	AVERAGE TOTAL VIABLE COUNT PER TUBE PER DAY	NUMBER OF COLONIES DEVELOPING ON DUPLICATE ITACONATE PLATES	PRESENCE (+) OR ABSENCE (−) OF TURBIDITY 2 DAYS AFTER SAMPLING	NO. OF TUBES WITH MUTANTS IN EACH SUCCEEDING PERIOD[a]
0	2.4×10^4	2.7×10^4	1, 1	−	
	2.8×10^4		0, 0	−	0
	3.0×10^4		1, 0	−	
1	2.5×10^6	2.5×10^6	1, 0	−	
	2.5×10^6		1, 1	−	3
	2.5×10^6		0, 1	−	
2	6.2×10^6	1.0×10^7	1, 0	−	
	1.3×10^7		1, 0	−	12
	1.1×10^7		3, 1	−	
3	1.2×10^7	7.8×10^6	0, 1	−	
	3.5×10^6		0, 1	+[b]	4
	3.5×10^6		1, 0	−	
4	5.8×10^6	6.2×10^6	++,[d] ++[d]	+[b]	
	7.1×10^6		++,[d] ++[d]	+[b]	1
	6.2×10^6		0, 0	−	
5	1.6×10^6	2.2×10^6	0, 0	−	
	2.2×10^6		0, 0	−.	1
	2.7×10^6		0, 0	−	
6	1.6×10^6	2.8×10^6	3, 0	−	
	4.0×10^6		1, 0	−	1
	2.8×10^6		2, 1	−	
7	−[c]	−	−	−	3
8	8.8×10^6	8.4×10^6	0, 1	−	
	9.6×10^6		3, 2	+[b]	2
	8.0×10^6		3, 1	−	
9	1.5×10^7		++,[d] ++[d]	+[b]	
	1.1×10^7		++,[d] ++[d]	+[b]	3
	1.1×10^7		++,[d] ++[d]	+[b]	
10	1.7×10^7	1.2×10^7	++,[d] ++[d]	+[b]	
	1.2×10^7		3, 1	−	2

[a] The time at which mutation occurred is equal to the time in days when turbidity was observed less 2 days.

[b] The viable count of this tube was omitted in calculating the average viable count since the tube contained mutants at the time of sampling.

[c] No viable counts performed.

[d] Growth too heavy to count.

that any observed increase in the total viable count of a tube prior to the appearance of visible turbidity had not resulted from early mutant growth. As an added check on this point, the tubes used for assay were always reincubated for several days after sampling, since had mutants been present at the time of assay they would have given rise to visible turbidity within 48 hours. This control is

important, since the slight growth of the parent on mineral itaconate agar plates sometimes results in the appearance of a few (1 to 5) itaconate-positive colonies. Such positive plates were disregarded if the remainder of the assayed tube failed to become turbid within 48 hours.
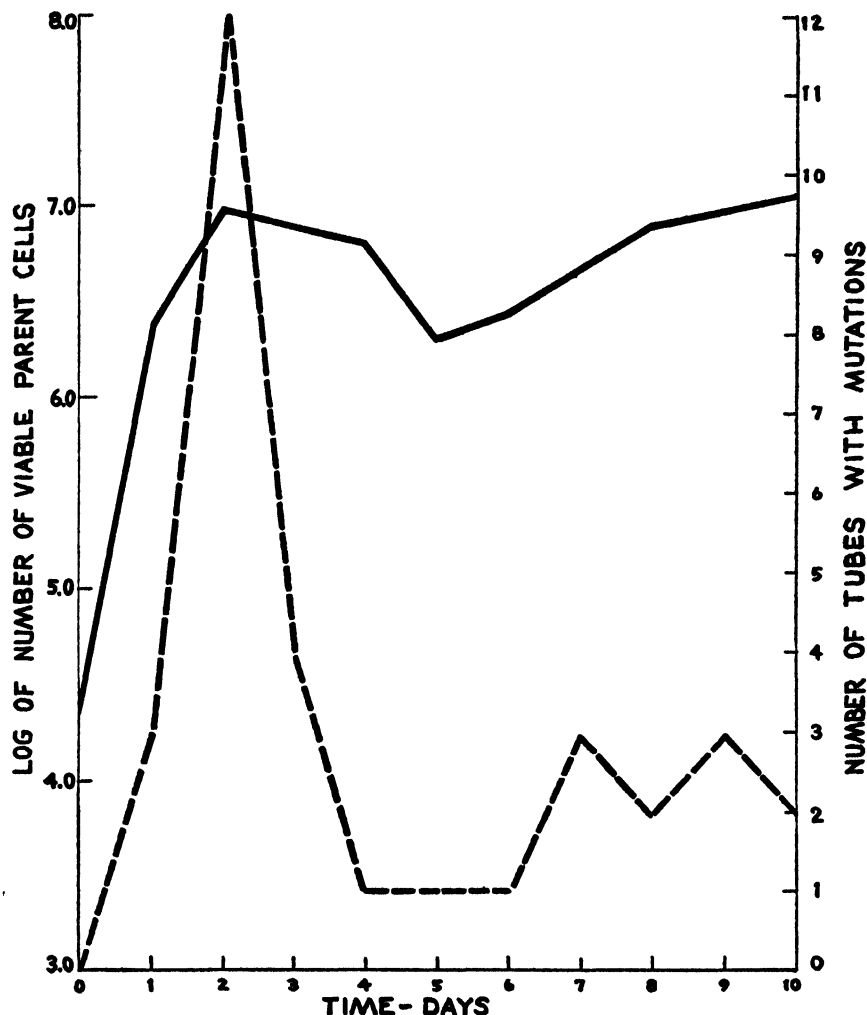
Figure 1. The relationship between growth and mutation (data from experiment 4). Solid line indicates viable count; broken line, number of mutants.

The number of positive tubes was also recorded daily, and differential counts on such tubes were made in order to establish the fact that the turbidity had resulted from growth of the mutant. The time of occurrence of the mutation was calculated again by subtracting 2 days from the age of the culture at the time when turbidity became visible.

As shown in table 2 and figure 1, extensive growth of the parent actually

TABLE 3
The relationship between growth and mutation
(Experiment no. 5)

DAYS AFTER START OF EXPERIMENT	TOTAL VIABLE COUNT PER TUBE	AVERAGE TOTAL VIABLE COUNT PER TUBE PER DAY	NUMBER OF COLONIES DEVELOPING ON DUPLICATE ITACONATE PLATES	PRESENCE (+) OR ABSENCE (−) OF TURBIDITY 2 DAYS AFTER SAMPLING	NO. OF TUBES WITH MUTANTS IN EACH SUCCEEDING PERIOD[a]
0	1.0×10^4	1.0×10^4	0, 0	−	
	8.9×10^3		0, 0	−	0
	1.1×10^4		0, 0	−	
1	2.1×10^6	3.1×10^6	1, 2	−	
	2.7×10^6		3, 1	−	4
	4.4×10^6		0, 2	−	
2	1.7×10^6	1.9×10^6	2, 5	−	
	1.6×10^6		3, 4	−	0
	2.3×10^6		0, 2	−	
3	2.2×10^6	1.9×10^6	3, 1	−	
	2.2×10^6		0, 2	−	1
	1.3×10^6		3, 3	−	
4	1.8×10^6	2.0×10^6	0, 3	−	
	2.1×10^6		1, 1	−	0
	3.3×10^6		++,d ++d	+b	
5	3.2×10^6	3.1×10^6	2, 0	−	
	2.8×10^6		3, 1	−	3
	3.2×10^6		1, 1	−	
6	2.9×10^6	3.6×10^6	0, 0	−	
	4.3×10^6		0, 0	−	2
	3.3×10^7		++,d ++d	+b	
7	3.7×10^6	6.1×10^6	0, 1	−	
	7.1×10^6		−	−	1
	7.5×10^6		1, 1	−	
8	4.4×10^6	4.7×10^6	2, 1	−	
	4.9×10^6		0, 0	−	2
	4.8×10^6		1, 1	−	
9	3.6×10^7	6.8×10^6	2, 1	+b	
	7.3×10^6		0, 1	−	1
	6.3×10^6		1, 2	−	
10	−c	−	−	−	2
11	2.6×10^7	1.5×10^7	++,d ++d	+b	
	1.5×10^7		0, 1	−	2
	1.4×10^7		0, 1	−	
12	−	−	−	−	3
13	1.2×10^7	2.7×10^7	1, 1	−	
	4.1×10^7		1, 0	−	1
	9.6×10^7		2, 1	+b	

[a] The time at which mutation occurred is equal to the time in days when turbidity was observed less 2 days.

[b] The viable count of this tube was omitted in calculating the average viable count since the tube contained mutants at the time of sampling.

[c] No viable counts performed.

[d] Growth too heavy to count.

occurs in such conditions. During the first 2 days, the parent population increased from 2.7×10^4 to 1.0×10^7 cells per tube, and concomitantly with this increase a large number of mutations occurred. Thereafter the total population remained more or less stationary for several days, during which time the number of new mutations was very small. After 5 days there was a slow secondary rise in the parent population, and this was once more reflected by an increased occurrence of mutations.

In an attempt to reduce the amount of growth of the parent, the experiment was repeated under more rigorous conditions. Pure doubly distilled water was used for the preparation of the medium (medium 3a), and 0.001 per cent $FeCl_3$ was added to the mineral base. Cotton plugs were completely eliminated, and

TABLE 4

Determination of mutation rate

EXPT. NO.	VIABLE COUNT (N)	TIME OF OCCUR-RENCE OF MU-TATION	NO. OF CULTURES WITH MUTANTS (x)	TOTAL NO. OF SIMILAR CULTURES (C)*	MUTATION RATE† (a)
		days			
4	2.5×10^6	1	3	54	1.6×10^{-8}
4	1.0×10^7	2	15	54	2.3×10^{-8}
5	3.1×10^6	1	4	54	1.7×10^{-8}
Average.............					1.9×10^{-8}

* The total number of similar cultures is determined by subtracting the number of tubes used for the determination of the viable counts from the original number of tubes employed, since the former tubes can no longer be considered similar to the remaining ones. In experiment 4, in order to calculate the mutation rate for the second day of the experiment, C_0 was taken to be 54 although 3 of the tubes were previously employed in determination of the mutation rate. This procedure introduces a negligible error into the final calculation.

† The equation employed in calculating the mutation rate is that of Luria and Delbrück (1943),

$$a = -\frac{\ln 2 \times \ln \frac{C_0}{C}}{N}. \quad C_0 \text{ equals } C \text{ less } x.$$

replaced by inverted glass vials to protect the tubes from contamination. The results of this experiment, as shown in table 3, are very similar to those of the previous one, the initial rapid rise in parent population being decreased only slightly in magnitude. As a check on the amount of growth that resulted from impurities present in the one organic component of the medium, daily total viable counts were also made on similar tubes containing the mineral base alone without itaconate. Even under these circumstances the population per tube increased from 1.2×10^4 to 4.0×10^5 cells during the first 2 days. Thereafter, however, it remained stationary for 13 days, the later slow secondary rise in the parent population observable in the presence of itaconate never occurring. We are unable to offer an explanation for this slight, but unmistakable, secondary rise in the

presence of itaconate. Evaporation was not a cause, as shown by volume determinations on selected tubes. The fact that it is separated from the initial rapid rise by a period of several days suggests that it may result from the mutational acquisition of the ability to attack itaconate very slowly; if so, the acquired biochemical ability is too weak to permit assay of these mutants on itaconate agar plates.

Determination of the mutation rate. The foregoing experiments show that a close relationship exists between parental growth and mutation and that if astronomical time is a factor at all in the appearance of mutants, it is one of negligible magnitude. This being the case, a calculation of mutation rate as defined by Luria and Delbrück (1943), on the basis of a physiological time unit, appears legitimate. All the necessary data are present in experiments 4 and 5. The results of three calculations, two derived from the former and one from the latter experiment, are given in table 4. The similarity of the figures obtained provides additional evidence for the hypothesis that growth is the chief factor controlling the appearance of the mutant in these experiments.

DISCUSSION

In the past it has frequently been assumed that absence of visible turbidity in a liquid medium implies absence of growth. Since a bacterial population density of 10^7 to 10^8 cells per ml is required to produce visible turbidity, this assumption is clearly unjustified in situations in which the milieu is marginal, and a failure to realize its invalidity can lead to highly erroneous conclusions. Our experiments have shown that *Pseudomonas fluorescens*, a typical bacterial autotroph, can grow for many generations to population densities below the visible level in a medium devoid of any deliberately added utilizable carbon source. This observation, although at first sight surprising, is thoroughly understandable if one makes a simple calculation. The only factor limiting development is the absence of an oxidizable substrate, which for *Pseudomonas fluorescens* can be supplied by any one of a wide variety of simple organic compounds (Den Dooren de Jong, 1926). The weight of oxidizable carbon required for growth from a single cell to 10^7 cells (assuming 30 per cent assimilation) is 2 μg, which would correspond for a carbohydrate to 5 μg of oxidizable substrate. Such small amounts of oxidizable organic material could easily be introduced in the ordinary cp chemicals employed for preparing media. The same situation would apply for any bacterial autotroph.

The possibility of extensive "invisible" growth is an important factor in experiments on the genetics of bacteria. The fact that mutations take place in media formally devoid of a utilizable substrate cannot be taken as prima facie evidence for spontaneous mutation in a nonproliferating population. In fact, the analysis of the occurrence of itaconate mutants of *Pseudomonas fluorescens* under such circumstances has shown that the frequency of mutation is closely correlated with the degree of invisible parental growth. Since the biological and environmental factors in the experiments of Lwoff and Audureau (1941) on the succinate mutant of *Moraxella lwoffi* were analogous to ours, it now appears highly probable that

this mutation is also growth-linked. At all events, their claim that the succinate mutant arises in the absence of parental growth should not be accepted without a much closer experimental analysis. It may be pointed out that with autotrophs such as *Pseudomonas fluorescens* and *Moraxella lwoffi* experimental conditions in which parental growth can be rigorously excluded will probably be extremely difficult to devise.

When spontaneous mutations are expressed phenotypically by the ability to utilize a substrate that the parent cannot attack, a very simple experimental procedure may be used to calculate the mutation rate. A series of identical liquid cultures containing the substrate in question as the sole added carbon source is prepared. Invisible growth of the parent at the expense of impurities will occur up to the point at which a mutation becomes probable. Once mutation in a tube occurs, a mutant population will develop rapidly and carry the population density well over the level of visible turbidity, making it possible to detect mutation by simple inspection. By determining the total viable population and the proportion of tubes with mutants at one given time, the rate can be calculated from the formula of Luria and Delbrück. The same experiment also constitutes, of course, a fluctuation test for the random origin of the phenotypic change observed.

This simplified procedure eliminates the considerable experimental difficulties involved in the extension of Luria and Delbrück's (1943) methods (designed for the determination of rates of mutations from virus sensitivity to virus resistance) to the determination of the rates of positive biochemical mutations. In order to determine a mutation rate it is necessary to relate the number of mutations or the number of mutants to the viable count of the entire population. In the procedure of Luria and Delbrück (1943) the number of mutations or the number of mutants is determined by several platings from each of a series of similar cultures onto an agar medium upon which only the mutant can grow. Although this procedure works perfectly well for mutations to virus resistance, since the growth of the parent on the mutant assay plates is eliminated by the presence of the virus, it is unreliable when applied to the study of positive biochemical mutants. In the latter case it is difficult to make the medium rigorously selective, and considerable growth of the parent is likely to occur on the mutant assay plates. As a result, one cannot determine whether the mutants that develop were initially present in the tubes or whether they originated on the plates during growth of the parent. Obviously, therefore, the viable count per tube cannot be related with any accuracy to the mutants appearing on the plates. For this reason the direct application of Luria and Delbrück's methods for the determination of mutation rates to positive biochemical mutations is open to question. Furthermore, even if the growth of the parent on the medium used to count the mutant could be reduced to an insignificant value, our simplified method eliminates the entire necessity of plating to determine the presence of mutation.

Our proposed technique could be used with slight modification for determining the rates of spontaneous mutations involving acquisition of the ability to synthesize a particular growth factor. Fildes and Whitaker (1948) have shown that the

growth of tryptophan-requiring *Salmonella typhosa* can be kept at subvisible levels by low tryptophan concentrations that still permit sufficient growth for mutations to tryptophan independence to occur. Once such a mutation takes place, visible turbidity from growth of the mutant soon results. Thus the necessary conditions for simple determinations of mutation rates in this and similar cases can be established by controlling closely the amount of the crucial growth factor supplied.

SUMMARY

Extensive "invisible" growth of *Pseudomonas fluorescens* to a level below that causing turbidity can occur in a medium not containing any known added utilisable carbon source.

There is a high positive correlation between such growth and the appearance of spontaneous mutants.

In the case of spontaneous mutation involving the acquisition of a new biochemical function, a very simple method for the calculation of mutation rate, based upon this limited parental growth, can be used.

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THE CONSEQUENCES OF MUTATION DURING THE GROWTH OF BIOCHEMICAL MUTANTS OF ESCHERICHIA COLI

II. THE INHIBITION OF HISTIDINE-INDEPENDENT BACTERIA BY HISTIDINELESS BACTERIA IN UNSHAKEN CULTURES[1]

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During the growth of a histidineless mutant (h−) of *Escherichia coli* there appear, with a low frequency, histidine-independent (h+) back mutants (Ryan, 1948). These h+ back mutants grow in the absence of histidine at the same rate and to the same extent as do the h− parents when supplied with optimal concentrations of histidine. Unlike their h− parents, these h+ back mutants, when grown by themselves, behave in a way that is independent of the histidine content of the medium. In the presence of optimal concentrations of histidine there is no selective advantage to synthesize this compound. Mixtures of h+ and h− organisms, when grown under such conditions, retain their initial proportion for many generations. In the absence of histidine, on the other hand, there is a selective advantage to synthesize this substance, and h+ bacteria will overgrow the population of h− organisms from which they were derived.

On intermediate, limiting concentrations of histidine h+ bacteria can undergo an amount of growth that is limited by the number of h− bacteria present in the culture (Ryan and Schneider, 1948). As a consequence, adaptation, the overgrowth of an h− culture by h+ organisms produced within it, becomes progressively less as the histidine concentration increases. In some way the h− organisms, when grown on suboptimal concentrations of histidine, can prevent the full growth of h+ bacteria. The mechanism of this action is the subject of the present and the following papers (Ryan and Schneider, 1949a,b).

EXPERIMENTAL PROCEDURES

The material and most of the methods used in this study have already been described (Ryan and Schneider, 1948). In addition, hydrogen ion concentrations were determined with a Beckmann model H pH meter. The pH of the medium was determined after autoclaving, and modifications were made by the addition of either sodium hydroxide or hydrochloric acid. When the pH of a filtrate, or of a whole culture, was to be raised, a determination of the amount of sterile alkali required was first made on an aliquot. Total additions to the cultures were always less than 10 per cent of the original volume. Sterile filtrates were prepared by passage through pyrex UF sintered glass crucibles. All experiments reported in this paper were run separately at least twice; most of them were run many times.

All cultures, unless otherwise mentioned, were kept stationary. They were not, strictly speaking, unagitated. Before turbidity determinations were made each culture was spun by hand to make the suspension as homogeneous as possible. The number of turbidity determinations and the amount of spinning was an uncontrolled variable in these experiments. However, the cultures were not continuously shaken as was the case in experiments to be reported later (Ryan and Schneider, 1949a).
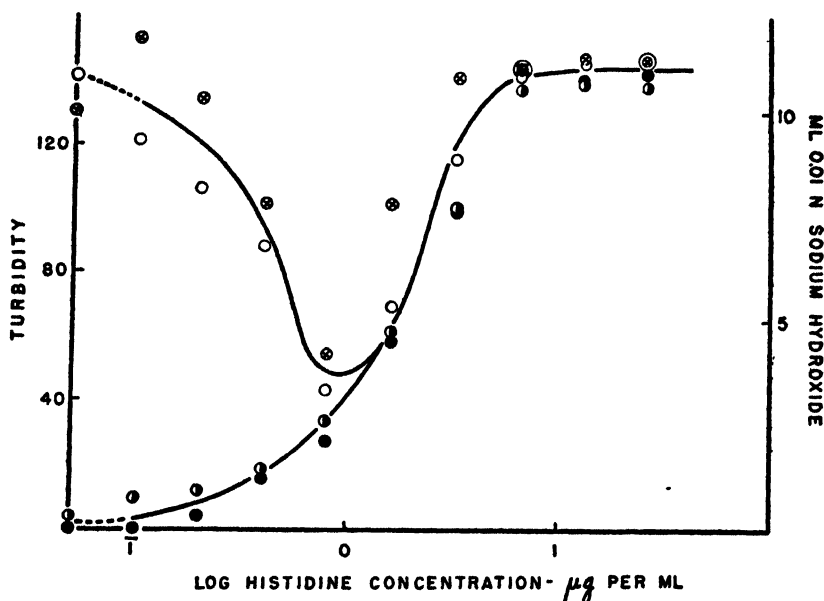

Figure 1. The parallel behavior of growth and acid produced by h— cultures on different concentrations of histidine. The solid circles are for unadapted growth at 14 hours and the half-open circles for the amounts of 0.01 N sodium hydroxide required to bring the cell-free filtrates of the unadapted cultures to a pH of 7.0. The open circles are for adapted growth (38 hours) and the crossed circles for the base equivalents of the acidity of filtrates of the adapted cultures. The dotted lines go to the levels of growth and acidities on minimal medium devoid of histidine.

RESULTS

The limitation of growth. Figure 1 shows the levels of growth attained before and after the adaptation of 19 ml h— cultures in unshaken test tubes. During growth, acid is produced in amounts proportional to the number of cells that are formed; the titratable acidity is shown, in figure 1, to parallel the levels of growth before and after adaptation. As a consequence, the pH of the culture medium is reduced to the varying extents shown in figure 2. Where growth is least after adaptation, there the pH has been reduced to the least extent. The level of adap- tive growth is stable and does not change for a period of several weeks, but the pH continues to drop, reaching a limit of about 5.5. Yet these pH's, even shortly after adaptation when they may be as high as 6.5, limit growth on intermediate concentrations of histidine. This has been shown in two ways. In the first place,

when whole h— cultures were allowed to adapt on a medium containing 0.4 or 0.8 µg histidine per ml and several hours later were brought to a pH of 7 (with sterile sodium hydroxide or sterile mixtures of sodium or potassium phosphate), growth was reinitiated, although at a slow rate. During the same period no growth at all was shown by controls that were allowed to remain at the pH produced by adaptation. Similarly, when sterile filtrates of h— cultures, made sometime after adaptation on 0.4 and 0.8 µg histidine per ml, were inoculated with h— or h+ organisms, no growth occurred unless inoculation was preceded by the sterile neutralization of the filtrates. By neither technique could growth be obtained with the addition of histidine, sugar, or any of the mineral constit-
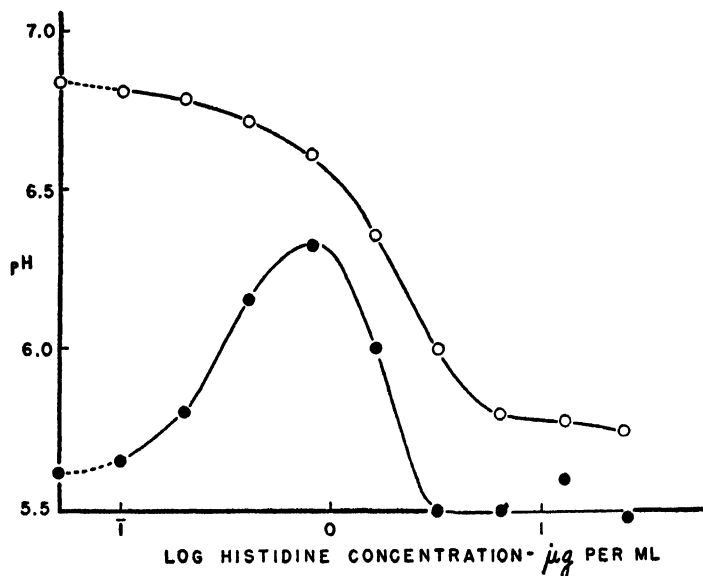

Figure 2. The pH of whole unadapted (14-hour) and adapted (38-hour) h— cultures as a function of histidine concentration. The upper curve is for unadapted and the lower curve for adapted cultures. The dotted lines go to the pH's produced on minimal medium devoid of histidine.

uents of minimal medium singly or in combination, provided that the pH was not raised. It may be concluded that pH limits the poor growth obtained at the dip in the adaptation curve.

Since this is so, an increase in the buffer capacity of the medium should, by resisting the effects of acid production, allow for greater growth. The minimal medium used contains 3 g K_2HPO_4 and 1 g KH_2PO_4 per liter and, after autoclaving, has a pH of about 6.8. Figure 3 shows the effect of increasing the concentration of these phosphates to various extents, yet always maintaining the 3:1 ratio. In the range between 0.4 and 0.8 µg of histidine per ml, where the adaptation curve is depressed, a striking effect is noticed. Considerably more growth is achieved on double and quadruple phosphate medium than on single-strength medium. (Eight- and sixteenfold increases in phosphate appear to be toxic.) Similarly the amount of growth achieved on lower concentrations of histidine or
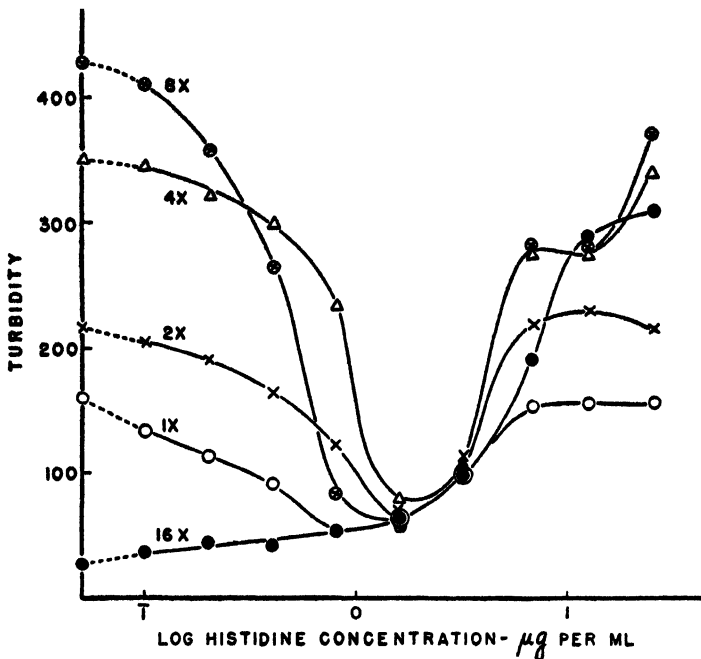
Figure 3. The level of adaptive growth attained by h— cultures after 46 hours on different concentrations of histidine in medium containing different amounts of phosphate. The numbers on the curves refer to the concentrations of phosphate as described in the text. The dotted lines go to the levels of growth on medium devoid of histidine.
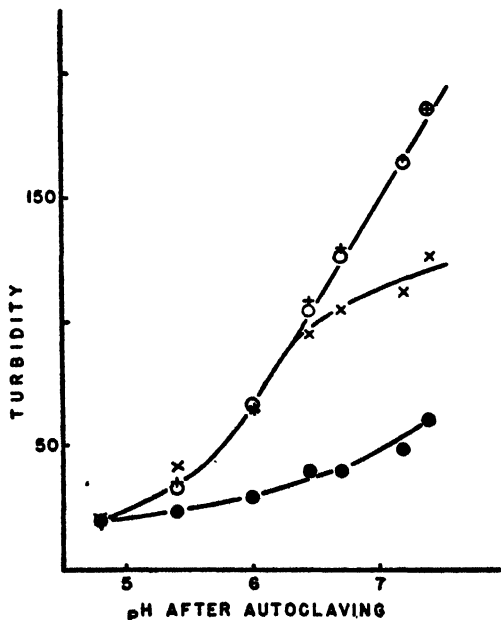

Figure 4. The effect of initial pH (after autoclaving) on the stationary level of preadaptive growth of h+ and h— cultures in the presence of different concentrations of histidine. The open circles enclose points representing the level of growth of h+ bacteria after 16 hours in the presence of 0, 0.4, and 3.2 μg histidine per ml. The solid circles, X's, and +'s refer to the levels of growth attained by h— bacteria in the presence of 0.4, 3.2, and 25 μg histidine per ml, respectively.

in its absence and on optimal concentrations of histidine is greater the larger the buffer capacity of the medium. In the presence of 1.6 and 3.2 μg histidine per ml, however, the amount of growth is independent of the buffer capacity, and on the different media the same amounts of growth reduced the pH to different levels between 6.8 to 6.5. Thus, on low and on optimal concentrations of histidine the amount of buffer, probably through its effect in resisting the decrease in pH, seems to determine the final level of adaptive growth; but on 1.6 and 3.2 μg histidine per ml some other factor is involved.

In order to test this point the amount of growth of h− and h+ cultures was determined on different concentrations of histidine at different initial pH's
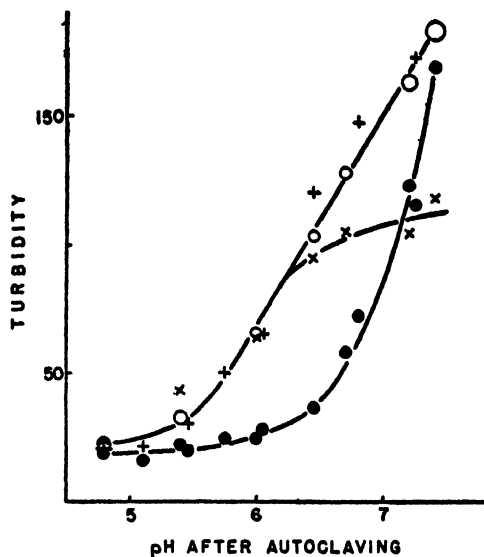

Figure 5. The effect of initial pH (after autoclaving) on the final adaptive level of growth of h+ and h− cultures in the presence of different concentrations of histidine. The open circles enclose points representing the level of growth of h+ bacteria after 38 hours in the presence of 0, 0.4, and 3.2 μg histidine per ml. The solid circles, X's, and +'s refer to the levels of growth attained by h− bacteria in the presence of 0.4, 3.2, and 25 μg histidine per ml, respectively.

(figures 4 and 5). Cultures of h+ bacteria, irrespective of the histidine concentration they contained, showed a regular increase in growth with increase in pH. The same sort of increase was exhibited by h− cultures when grown on an optimal (25 μg per ml) concentration of histidine. Before adaptation, in the presence of 0.4 μg per ml, there was also an increase in growth with a rise in pH, but at any pH the growth was less than that achieved on optimal histidine concentrations. After adaptation there is a sharp increase in the levels of growth above pH 6.5 due to the multiplication of the small number of h+ mutants in the h− culture. Below pH 6.5, however, h+ mutants do not overgrow the culture and adaptation does not occur. Obviously the h+ bacteria are influenced in their behavior by the h− organisms present. In the presence of 3.2 μg histidine adaptation did not occur at any of the pH's studied (4.8 to 7.4) despite the fact that live

h+ bacteria were shown (by plating) to be present. Further, on this concentration the total growth was approximately the same at pH 6.5 and 7. This finding is consistent with the results obtained with media buffered to different extents. But it is still necessary to explain why h+ organisms at an initial pH of 6.5 can grow to an optical turbidity of about 100, although in the presence of h− organisms they are prevented from growing at a pH of 6.5 and adaptation does not occur.

Test of inhibition. This restriction in the growth potential of the h+ organisms could be due to the addition to, or the removal from, the medium of something by the h− bacteria. If the latter were true, then growth should be reinitiated by the addition of fresh medium (unless, of course, the restriction involved a permanent change). A series of h− cultures were allowed to adapt on histidine concentrations from 0 to 25 µg per ml. At this time solutions of salts, sugar, and histidine, singly and in combination, were brought to the pH of the adapted cultures and added to them. The volumes added were one-tenth of the volumes of the cultures, and the amounts of solute were ten times that in fresh medium containing 25 µg histidine per ml. Despite this, no new growth ensued. The limitation of growth evidently was not due to the removal of something from the medium.

Consequently something that we can call an inhibitor or inhibitors must have been added by the h− bacteria. There are two simple hypotheses regarding the production of an inhibitor by h− bacteria that would explain the dip in the adaptation curve. The first assumes that h− bacteria produce an inhibitor only on limiting concentrations of histidine. In this event filtrates from cultures grown on such concentrations should be the least favorable for new growth. The second hypothesis assumes that the h− bacteria produce an inhibitor in proportion to their number and that this inhibitor is specific against h+ bacteria. In this event filtrates from cultures grown on optimal concentrations of histidine should be the least favorable. In order to discriminate between these hypotheses sterile filtrates were prepared of h− cultures allowed to adapt on different histidine concentrations. These were neutralized; aliquots were kept in this form or were supplemented with optimal amounts of histidine and were then inoculated with h+ or h− bacteria. This experiment was performed four times and the results were never the same. All experiments, however, had some features in common. First, unneutralized control filtrates supported no growth after inoculation. Second, filtrates from some histidine concentrations supported the growth of fewer bacteria than others. The most unfavorable filtrates, however, came, in one experiment, from cultures grown on 0.4 µg histidine per ml and, in another, from cultures grown on 25.6 µg per ml. Further, when the adapted filtrates were neutralized and mixed with equal volumes of double-strength fresh medium with or without histidine, results of the same sort were obtained. Some of the filtrate experiments performed seemed consistent with the first hypothesis, but the remainder were inconsistent with it. The remainder were also inconsistent with the second hypothesis because filtrates from cultures that supported the growth of large numbers of h− organisms were equally unfavorable for the growth of h−

and h+ bacteria. There was no evidence that the filtrates contained inhibitors specific against h+ organisms.

In order to test the inhibition hypothesis further, filtrates were assayed for their effect on the rate of growth of cultures maintained in the logarithmic phase by a method described in the following paper (Ryan and Schneider, 1949a). The filtrates were neutralized, concentrated under vacuum, and added in volumes not over 10 per cent of the total to cultures of h− and h+ bacteria in the logarithmic phase of growth. Figure 6 shows the effect of adding a filtrate from an
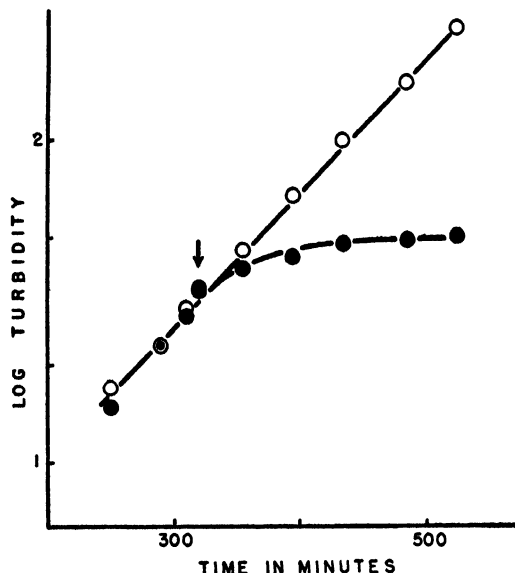

Figure 6. The inhibition of the rate of growth of h+ bacteria by a filtrate of a culture of h− bacteria. To the left are shown the growth curves of two shaken cultures of h+ bacteria in minimal medium. At the time indicated by the arrow one culture received a sterile concentrate of uninoculated minimal medium evaporated from 10 to 1 ml. The upper curve shows the relatively unimpaired growth of the h+ bacteria which followed this addition. The other culture received at the same time a sterile concentrate of a filtrate of an unshaken culture of h− bacteria grown for 19 hours in the presence of 25 μg histidine per ml and evaporated from 10 to 1 ml. The lower curve shows the inhibited growth of the h+ bacteria following this addition.

h− culture to h+ bacteria. The rate of growth was decreased to about 20 per cent of the initial value. On the other hand, the addition of concentrated minimal medium did not appreciably affect the rate of growth. Such behavior on the part of controls was consistent; in eight experiments the rate of growth after the addition of concentrated minimal medium was 106 ±14 per cent of the rate before addition. That the concentration of the filtrate did not in itself produce the inhibitor(s) was shown by experiments in which filtrates from h− cultures grown in the presence of 25 μg histidine per ml were concentrated, then restored to their initial volume. They were compared with aliquots of the same filtrate that had not been concentrated. The two types of preparations in several experiments gave inhibitions that were within 10 per cent of one another. Further, the inhibitory
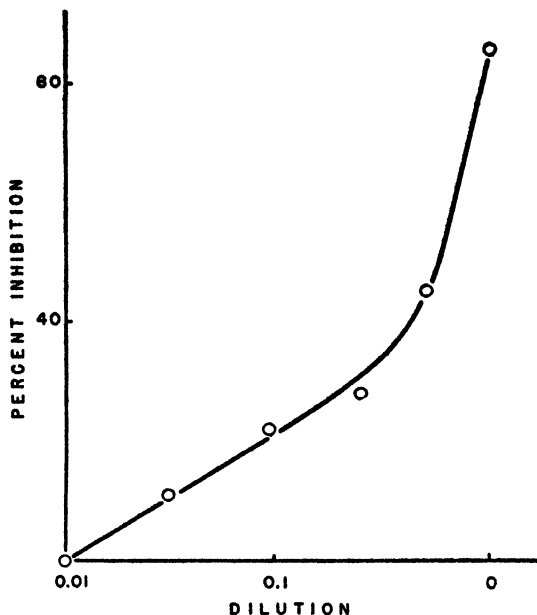
Figure 7. The effect of dilution on the inhibitory action of a filtrate of an h— culture on the rate of growth of h+ bacteria. The h— culture had been grown for 18 hours in the presence of 25 μg histidine per ml; it was then filtered and the filtrate was evaporated from 10 to 1 ml. The h+ culture was grown in shaken minimal medium and the percentage of inhibition was calculated from the change in the logarithmic rate of growth before and after the addition of the filtrates.
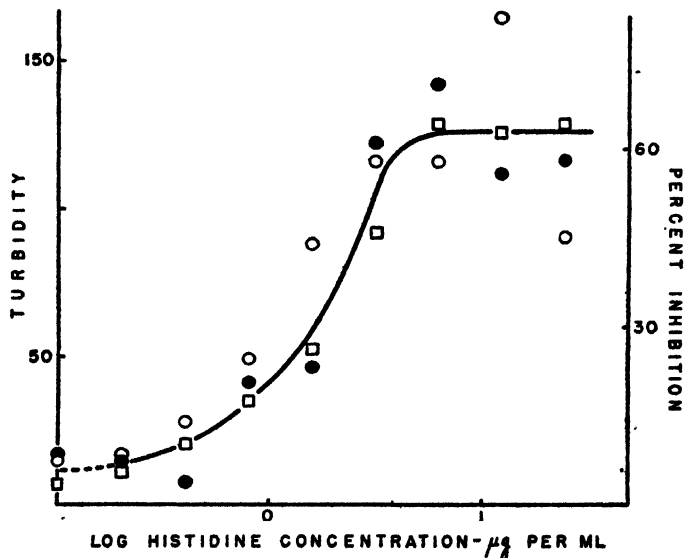
Figure 8. The extent of inhibition, by filtrates of unadapted h— bacteria grown on different concentrations of histidine, of the rate of growth of h— and h+ organisms. The abscissa denotes the histidine concentrations on which the h— bacteria were grown prior to filtration and concentration from 10 to 1 ml. The ordinate on the left measures the turbidity of these cultures. The ordinate on the right measures the percentage of inhibition as gauged by the difference in the logarithmic rate of growth of h+ and h— bacteria before and after the addition of the filtrates. The squares refer to the level of growth of the h— cultures after 19 hours just before filtration, the solid circles to the percentage of inhibition of h— bacteria grown in shaken medium supplemented with 25 μg histidine per ml, and the open circles to the percentage of inhibition of h+ bacteria grown in shaken minimal medium. The dotted line goes to points for filtrates of h— bacteria in the absence of histidine.

activity of the filtrate concentrates decreased upon dilution (figure 7) and would not stand complete desiccation or autoclaving for more than a few minutes.

Figure 8 shows the activities of filtrates of h— cultures grown in the presence of different concentrations of histidine. Although there is considerable scatter among the points, it is evident that the extent of inhibition of growth rate is proportional to the amount of growth that had ensued in the cultures from which the filtrates were prepared. In these experiments the final levels of growth of the cultures to which the filtrates had been added were decreased as much as 20 per cent when the rate of growth was slowest. Similarly, filtrates from adapted cultures showed inhibitions in proportion to the number of bacteria in the cultures from which they were made. Once again these findings are inconsistent with the hypothesis claiming the production of an inhibitor by h— bacteria only on intermediate concentrations of histidine where the dip in the adaptation curve occurs. Further, the filtrates were equally inhibitory to h+ and h— bacteria. Thus the inhibitor (or inhibitors) is not specific against the h+ back mutants and its action cannot explain, at least in a simple fashion, the depression of adaptation on intermediate concentrations of histidine. Indeed, filtrates from cultures of h+ bacteria show the same nonspecific inhibitory activity and to the same extent as filtrates from h— cultures containing similar numbers of bacteria. A later paper in this series will present evidence revealing the way in which the inhibitor (or inhibitors) depresses adaptation (Ryan and Schneider, 1949b).

SUMMARY

In unshaken cultures of *Escherichia coli*, in which the pH decreases in proportion to the number of bacteria, the adaptive growth of histidine-independent back mutants takes place until intolerable pH's arise. The pH tolerance of these back mutants is determined by the proportion of parental histidineless bacteria also present in the culture. This restriction by the histidineless bacteria is not brought about by the depletion of something from the medium but rather by the production of some nonspecific substance (or substances) that is formed in proportion to the number of bacteria. These results, in conjunction with those reported in the following paper (Ryan and Schneider, 1949a), are discussed in the final paper of this series (Ryan and Schneider, 1949b).

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THE CONSEQUENCES OF MUTATION DURING THE GROWTH OF BIOCHEMICAL MUTANTS OF ESCHERICHIA COLI

III. The Inhibition of Histidine-Independent Bacteria by Histidineless Bacteria in Aerated Cultures[1]

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The preceding paper in this series (Ryan and Schneider, 1949a) demonstrated that the adaptation of unshaken histidineless (h−) cultures is limited by the acid produced during growth. The various pH's developed at intermediate histidine concentrations prevent the further growth of the histidine-independent (h+) mutants produced in these cultures. The sensitivity of the h+ bacteria to the various pH's is conditioned by something produced by h− bacteria during their growth. It was thought that the acid production might be a function of the partially anaerobic metabolism that must occur in the depths of the 10 ml of medium that were contained within the 15-by-150-mm pyrex test tubes (Stokes, 1949). Aeration might prevent this acid production and allow the phenomenon of adaptation to be studied without the complication of a continually changing pH. For this reason an analysis of shaken cultures of h− bacteria was undertaken.

EXPERIMENTAL PROCEDURES

The majority of the methods used in this research are described in the first two papers of this series (Ryan and Schneider, 1948, 1949a). In addition, in order to create aerated conditions of growth, the 10-ml aliquots of medium were placed in 125-ml pyrex Erlenmeyer flasks to each of which had been sealed a calibrated pyrex test tube at an upward angle of about 30° at the side near the bottom. It was possible to shake and vigorously aerate the culture in the bottom of the flask and then, without removal from the system, to spill it into the side arm. In this position the tube was inserted into the adapter of a Klett-Summerson colorimeter for the measurement of optical density. We are indebted to Dr. R. Ballentine for suggesting this method. Before sterilization cotton plugs were inserted that had been covered with cheesecloth to prevent fibers from falling into the medium. After inoculation the flasks were shaken in a water bath at 37 C through an amplitude of 3 cm at an optimal rate of about 100 cycles per minute.

A variety of changes in the composition of the medium were made in order to develop optimal conditions for shaken growth. Since asparagine was found to shorten the lag period only slightly, it was omitted from the medium in all experiments reported in this paper. It was also found that sterile-filtered glucose or

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glucose autoclaved with the medium resulted in the same lag period, the same logarithmic rate of growth, and the same final crop of h+ or h− bacteria. This was also true when the concentration of the inorganic constituents of the medium was as described in the first paper of this series (Ryan and Schneider, 1948) or was halved. Similarly, shaking at from 80 to 120 cycles and through thrusts of from 1 to 3 cm did not influence growth differentially. Consequently, full-strength medium, autoclaved with glucose, was chosen as the standard for shaking under the conditions described.

The concentration of glucose, when varied from 0.025 to 0.5 per cent, did not influence the lag period or the logarithmic rate of growth of either h− or h+ bacteria. However, the total amount of glucose in the 10 ml of medium did have
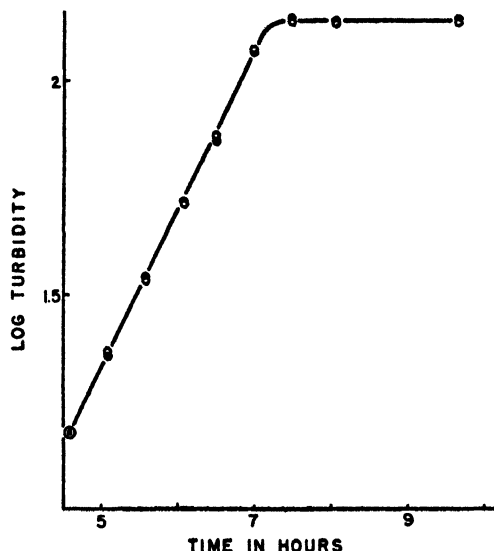
Figure 1. The growth curves of h+ and h− bacteria shaken in the absence of histidine and in the presence of 25 μg per ml, respectively. The open circles refer to h+ bacteria and the solid circles to h−.

a strong influence on the size of the final crop of bacteria. Moreover, it was found that at the completion of growth on 0.5 per cent glucose the pH of the medium dropped from 6.8 to about 4, despite aeration. On 0.05 per cent glucose the pH drops during growth from 6.8 to only about 6.5. Despite this small drop more than 5×10^8 bacteria are produced per ml. Apparently it is not aeration but sugar concentration that is critical in determining the amount of acid produced. Monod's (1942) medium, which also contains 0.05 per cent glucose, showed the same reduction in pH. It is, however, less well buffered and supported less bacterial growth. Consequently, we chose to use our medium with 0.05 per cent glucose.

RESULTS

Adaptation. Figure 1 shows the rate of growth of h+ bacteria in the absence of histidine and of h− bacteria in the presence of 25 μg per ml. The lag periods, loga-

rithmic rates of growth, and final populations of bacteria are the same. The final population size is limited by the amount of sugar present. When more sterile glucose is added in the stationary phase, growth is reinitiated. When, however, h— bacteria are grown on suboptimal concentrations of histidine, it is this substance that is limiting. When more sterile histidine is added to the culture, growth is reinitiated (figure 2).

The adaptation of h— cultures was studied on different concentrations of histidine under optimal aerated conditions. Figure 3 shows the time course of such
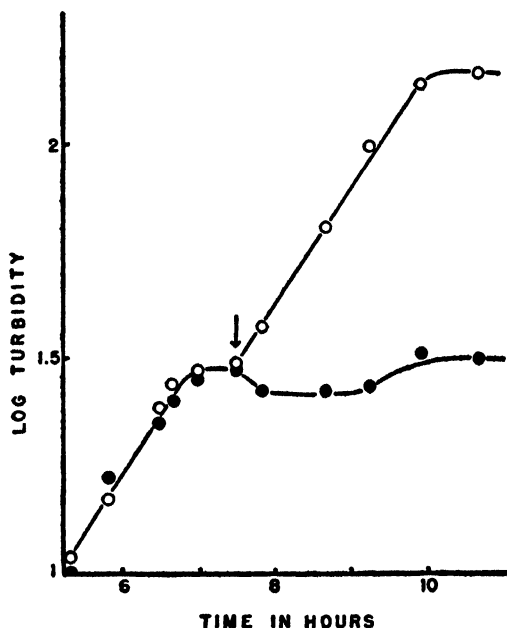

Figure 2. The effect of added histidine upon a shaken h— culture which had completed growth on a suboptimal concentration of histidine. Two cultures were allowed to grow in the presence of 0.8 μg histidine per ml until the stationary phase was achieved. At the time indicated by the arrow 0.1 ml of water, containing enough histidine to give a concentration of 25 μg per ml, was added aseptically to one culture. The control culture received just 0.1 ml of sterile water. The final growth achieved by the culture to which histidine was added is equal to the growth that the sugar in the medium would support.

adaptation. The rate of logarithmic growth is not dependent upon the initial concentration of histidine, but as this substance is depleted from the medium and becomes limiting, growth slows down and eventually stops. Those cultures that have received some histidine reach the stationary phase of growth within 9 hours. Then, after about 20 hours, the cultures on the lower concentrations of histidine and the one devoid of histidine begin adaptive growth, which is complete about 30 hours after inoculation. The time of adaptation is proportional to histidine concentration, as one would expect if the number of back mutations from h— to h+ were proportional to the number of h— bacteria present. The 30 hours required for the adaptation of the culture devoid of histidine is exactly the time required for the complete growth (5×10^{9}) of the 2 to 3 h+ bacteria
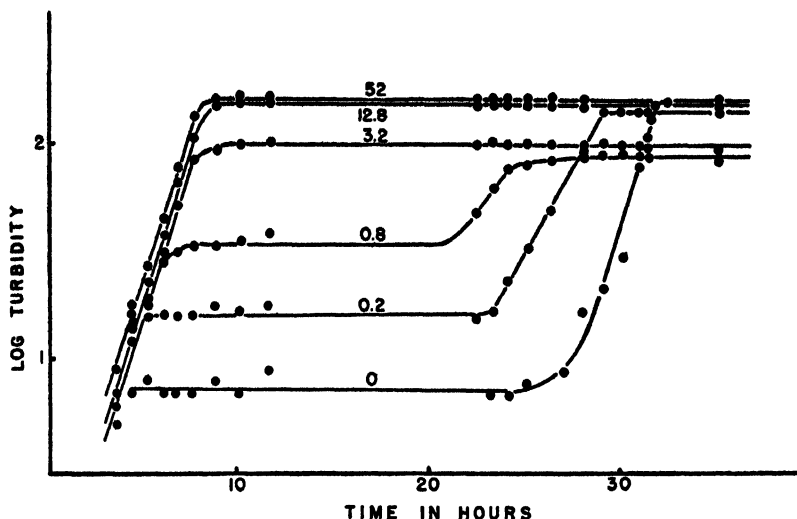
Figure 3. The time course of adaptation of shaken h— cultures on different concentrations of histidine. The numbers refer to the concentrations of histidine in μg per ml.
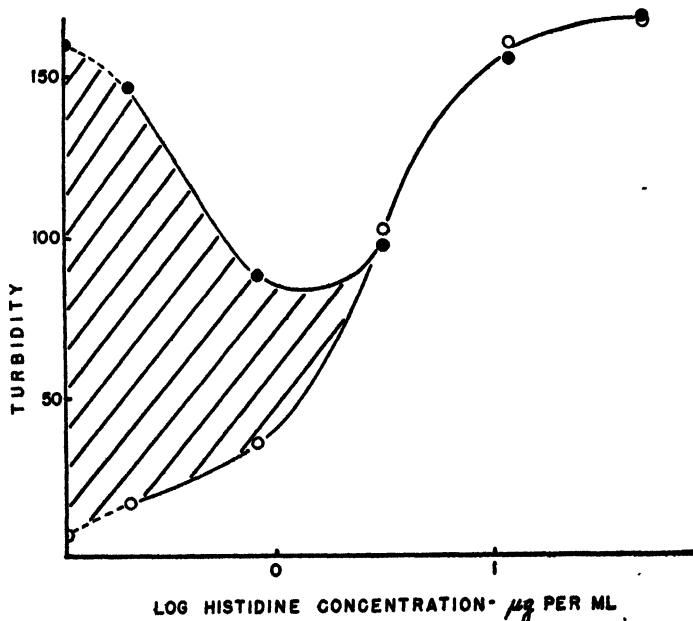
Figure 4. The relation of the amount of growth achieved with shaking before and after adaptation to histidine concentration. The lower curve is for growth at 10 hours and the upper curve for growth at 35 hours. The shaded area indicates the amount of adaptation. These data are the same as those shown in figure 3. The dotted lines go to the levels of growth on minimal medium devoid of histidine.

in the h— inoculum (2×10^7) if the generation time is the observed length of 55 minutes.

The extent to which adaptation takes place is, as it was in unshaken cultures, inversely proportional to the histidine concentration. Figure 4 shows this clearly.

Thus in aerated cultures, as in unaerated cultures, there is a dip in the adaptation curve, and the h+ bacteria, which are always present, do not grow to the extent expected. This depression of growth occurs despite the absence of the development of growth-limiting pH's in the course of growth. None of the aerated cultures had pH's of less than 6.5 after adaptation. Further, growth was not reinitiated by the sterile neutralization of any of these whole aerated cultures. Consequently, by shaking the cultures it is possible to study the inhibition of h+ by h− bacteria in the absence of the complication of the gradual development of a limiting pH during growth.

Test of inhibition. Filtrates suspected of containing inhibitors can be tested as they were in the studies reported in the previous paper (Ryan and Schneider, 1949a), by adding them to aerated cultures of h− and h+ bacteria in the logarithmic phase of growth. Sterile filtrates were prepared of unadapted and adapted

TABLE 1

The effect of filtrates of shaken h− cultures on the logarithmic rate of growth of
h+ bacteria expressed as percentage of the rate prior to the addition

H− CULTURE GROWN ON

μg Histidine per ml	For 18 hr unadapted	For 30 hr adapted
0	107	93
0.2	102	107
0.4	97	112
0.8	100	96
1.6	100	100
3.2	87	100
6.4	100	104
12.8	100	94
51.2	91	91

h− cultures grown under aerated conditions in the presence of 0.05 per cent glucose and different concentrations of histidine. These filtrates were neutralized, concentrated under vacuum, and added, in volumes not over 10 per cent of the total culture, to h+ organisms. These were in the logarithmic phase of growth in the absence of histidine under aerated conditions. Table 1 shows the effect of filtrates from h− cultures on the growth of h+ bacteria. Unlike filtrates from unshaken cultures no inhibitory action is evident. The difference is not entirely, if at all, due to the aeration, for aerated cultures containing 0.5 per cent glucose were shown by this technique to produce an inhibitor (or inhibitors). However, aerated cultures grown on 0.05 per cent glucose do not produce stable inhibitors of the growth rate of h+ bacteria.

In order to test the possibility that an inhibitor from cultures grown on 0.05 per cent glucose affects the final level of growth without influencing the rate of growth, filtrates of h− cultures grown on different concentrations of histidine were inoculated with h+ bacteria with and without further supplementation with glucose. Table 2 describes the results of such an experiment. There was essentially

no growth on the unsupplemented filtrates, but it occurred when sugar was added. However, this growth was independent of the amount of growth that had occurred in the culture from which the filtrate had been prepared. Hence once again there was no evidence for the presence of an inhibitor in the h— filtrates. It is further evident from this table that the new growth was to the extent expected on the basis of the sugar added. In other words, there was no sugar left in the

TABLE 2

The final turbidity of h+ bacteria grown for 14 hours in neutralized filtrates prepared from shaken h— cultures adapted on different concentrations of histidine

μg HISTIDINE PER ML ORIGINAL h— CULTURE	UNSUPPLEMENTED FILTRATE	FILTRATE SUPPLEMENTED WITH 5 MG STERILE GLUCOSE (0.05 PER CENT)
0	7	140
0.8	9	137
1.6	8	129
3.2	11	139
6.5	4	125
25.8	0	123

TABLE 3

The growth of adapted cultures of h— bacteria resuspended in fresh shaken medium containing 25 μg histidine per ml

μg HISTIDINE PER ML IN ORIGINAL CULTURE	OPTICAL TURBIDITY OF ORIGINAL ADAPTED CULTURE	h+ PER 10^6 h— IN ORIGINAL ADAPTED CULTURE	OPTICAL TURBIDITY AFTER RESUSPENSION	h+ PER 10^6 h— AFTER RESUSPENSION
0	132	$>10^7$	125	$>10^7$
0.1	125	$>10^7$	125	$>10^7$
0.2	123	$>10^7$	125	$>10^7$
0.4	97	$>10^7$	128	$>10^7$
0.8	64	4.2×10^6	126	4.7×10^6
1.6	70	1.1×10^6	126	1.4×10^6
3.2	107	25.0	125	37.0
6.4	132	0.57	123	0.49
12.8	136	0.56	120	0.61
25.6	140	0.62	122	0.50

filtrates of adapted h— cultures, and this despite the fact that they had supported different amounts of growth.

It is still possible that the h— bacteria produce an inhibitor (or inhibitors) that cannot be found in the filtrate because it is absorbed by the h+ organisms. In order to test this notion 31-hour-old adapted h— cultures, grown on different concentrations of histidine, were washed and resuspended in medium containing 25 μg histidine per ml. Table 3 shows the levels of growth achieved in the new medium and the h— and h+ composition of the culture at the time of resuspension and after the new growth. There is no indication that the h+ bacteria were inhibited in their growth.

Limitation of growth. In order to examine the inference that the exhaustion of glucose from the medium, rather than an inhibitor (or inhibitors), limits the extent of adaptive growth, direct chemical determinations were made of the glucose remaining in the filtrates of adapted h—cultures. However, considerable difficulty was encountered with these methods. The copper reduction method of Somogyi (1945) was found to be interfered with by NH_4^+ in the concentrations used in our minimal medium. The ferricyanide method of Folin and Malmros (Umbreit *et al.*, 1945) and the iodine method of Wilstätter and Schudel (Brown and Zerban, 1941), although they allowed accurate determination of the glucose in our minimal medium, were interfered with by something in the culture filtrates from which it was impossible to recover added glucose accurately. Consequently, the nonspecific yeast assay used by Spiegelman (1947) was adopted and found to work satisfactorily. Manometric measurement of the CO_2 production during anaerobic glycolysis of h— culture filtrates in the presence of azide by starved *Saccharomyces cerevisiae* allowed recoveries of added glucose which averaged 95 per cent complete. By this method, which can detect as little as 0.05 mg glucose per ml, no sugar was found present in filtrates of h— cultures adapted on different concentrations of histidine. This finding confirmed the bioassay of the filtrates by h+ bacteria. Further, by both biological methods, filtrates of unadapted h— cultures were shown to contain glucose in inverse proportion to the amount of growth that had taken place. Where growth was complete on high concentrations of histidine, no glucose could be detected, although on lower histidine concentrations in which less and less growth occurred more and more glucose remained. It is this remaining glucose that is available for the adaptive growth of the h+ back mutants.

If these determinations are correct and adaptive growth in aerated cultures is limited by the exhaustion of glucose from the medium, then the addition of glucose to adapted cultures should reinitiate growth. Figure 5, curve 1, shows that sugar does reinitiate growth and that it was limiting at most histidine concentrations. However, at the shoulder of the adaptation curve, in the region of 3.2 µg histidine per ml, new growth does not occur and something else appears limiting. The addition of a second allotment of fresh sugar (curve 2) shows that this is not true, for growth is then reinitiated. But the second sugar addition does not initiate new growth in the region of 6.4 µg histidine per ml.

This unusual state of affairs has a simple interpretation. After adaptation cultures on all histidine concentrations are limited by the absence of glucose. When glucose is added, however, it is only the h+ bacteria that will grow—unless there is some histidine left over. The cultures grown on limiting histidine concentrations have no histidine left over and after adaptation consist for the most part of h+ bacteria. When fresh glucose is added, these bacteria grow. The cultures in histidine concentrations that yield optimal growth do not use up the histidine present but are limited by the exhaustion of glucose. These cultures consist almost entirely of h— bacteria; only a few h+ back mutants are present because of the absence of selection for them. When fresh glucose is added, the h— bacteria begin to grow again by using the histidine not consumed during their

previous growth. The h+ back mutants also reinitiate growth and remain in their original proportion. On a concentration of histidine like 3.2 μg per ml histidine has been used up and is limiting. Nevertheless, adaptation does not occur, and the h+ bacteria are present in very small numbers. When fresh glucose is added, they begin to grow. The h− bacteria, which cannot grow, consume glucose in maintenance, and by the time the small number of h+ bacteria have grown to visible turbidity, the sugar is exhausted. The second addition of glucose, however, finds a large number of h+ bacteria present which rapidly grow to completion before the still stationary h− organisms have wasted much glucose. On the other
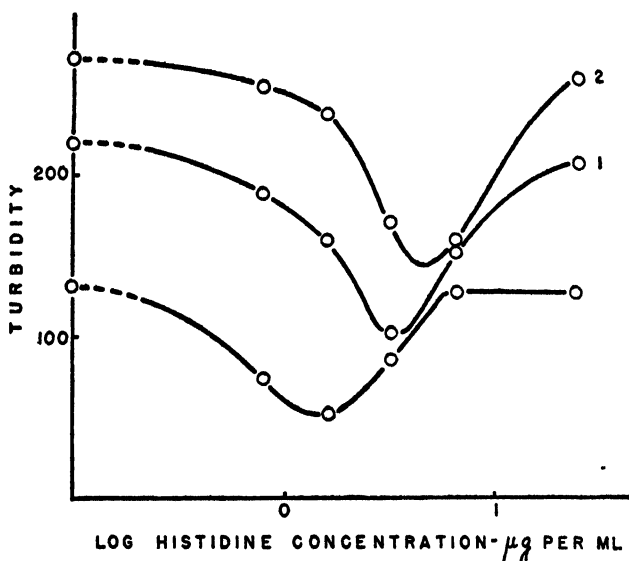

Figure 5. The effect of adding fresh glucose to shaken adapted cultures of h− bacteria grown on different concentrations of histidine. The lower curve shows the level of adaptive growth after 39 hours. At that time 5 mg sterile glucose (0.05 per cent) were added to each culture. Curve 1 shows the stationary level of new growth achieved 12 hours later. At that time another glucose addition was made and the new stationary level of growth achieved is shown by curve 2. The dotted lines go to the levels of growth on minimal medium devoid of histidine.

hand, the culture on 6.4 μg histidine per ml contains h− bacteria, and these grow, when the first sugar addition is made, until the excess histidine is exhausted. The small number of h+ back mutants present then begin to overgrow the culture, but before their proportion has increased appreciably the glucose is exhausted. The second glucose addition does not reinitiate growth of a culture started on 6.4 μg histidine for the same reason that the first addition did not reinitiate growth of the culture grown on 3.2 μg histidine per ml.

It will be evident that this hypothesis predicts the data shown in table 4. In the first place, on either side of the dip in the curve the new growth involves a different type of bacterium. On low concentrations of histidine, h+ bacteria, which were present in large numbers, overgrow. On high concentrations, in which there was an excess of histidine, the h− and h+ bacteria both grow, retaining

their proportion. On 3.2 μg histidine per ml the h— bacteria do not increase upon the addition of glucose, for the histidine has been exhausted. Indeed, there is some death (but not lysis) of the h— bacteria. But the h+ bacteria do grow although just to the level of visible turbidity (ca. 5×10^6 per ml). Hence the turbidity of the culture does not increase until a second glucose addition is made. In this way there is a depression in the curve after the addition of glucose despite the fact that all shaken adapted cultures are limited in growth by the absence of glucose.

In constructing this interpretation it was assumed as demonstrated that adapted cultures have used up their glucose supply. They do this without achieving the same amounts of growth. It is thus still necessary to account for this apparent paradox. If h— bacteria in the stationary phase consume glucose without adding to the cell population, it is possible to understand the depression in

TABLE 4

The composition of shaken adapted h— cultures before and 7 hours after the addition to each of 5 mg sterile glucose (0.05 per cent)

ORIGINAL HISTIDINE CONCENTRATION, μg PER ML	NUMBER OF BACTERIA PER ML				H+ PER 10^6 H—	
	Adapted culture after 35 hr		After growth on added glucose		Adapted culture after 35 hr	After growth on added glucose
	h+	h—!	h+	h—		
0	61×10^7	—	157×10^7	—	$>10^7$	$>10^7$
0.2	53×10^7	—	121×10^7	—	$>10^7$	$>10^7$
0.8	20×10^7	8×10^7	108×10^7	$<10^7$	2.5×10^6	$>10^7$
1.6	21×10^6	16×10^7	74×10^7	18×10^7	1.3×10^5	4.1×10^6
3.2	98×10^3	44×10^7	12×10^5	12×10^7	223	10^4
6.4	115	68×10^7	3×10^4	44×10^7	0.17	68
12.8	90	59×10^7	160	147×10^7	0.15	0.11
25.6	70	69×10^7	160	147×10^7	0.10	0.11

the adaptation curve and also all other facts that have been gathered in this connection. The following paper (Ryan and Schneider, 1949b) presents unequivocal evidence that this is the case.

SUMMARY

A method is described for the convenient measurement of the logarithmic rate of growth of *Escherichia coli* under conditions of shaking when the pH remains stable. Either the exhaustion of glucose or of histidine can be made to limit growth. Under these conditions adaptation of cultures of histidineless bacteria by the overgrowth of histidine-independent mutants occurs in the same way as in unshaken cultures. Similarly the adaptive growth of the histidine-independent bacteria is restricted by the presence of histidineless organisms on intermediate concentrations of histidine.

Unlike the situation in unshaken cultures, the production of inhibitory substances by the histidineless bacteria could not be demonstrated. Rather, the

growth of adapted cultures is limited by the exhaustion of glucose from the medium, and this despite the fact that unequal amounts of growth occurred on the different concentrations of histidine.

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THE CONSEQUENCES OF MUTATION DURING THE GROWTH OF BIOCHEMICAL MUTANTS OF ESCHERICHIA COLI

IV. THE MECHANISM OF INHIBITION OF HISTIDINE-INDEPENDENT BACTERIA BY HISTIDINELESS BACTERIA[1]

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The adaptation of a culture of histidineless (h−) *Escherichia coli* has been shown to be due to overgrowth by the small number of histidine-independent (h+) back mutants they contain (Ryan and Schneider, 1948). These back mutants are selected for when histidine is exhausted from the medium. Nevertheless, the adaptive growth of the h+ bacteria is not independent of the h− organisms in the culture, but rather its level is determined by their number. In unshaken cultures the level of adaptive growth is determined by a rather complex interaction between a falling pH and a rising concentration of an inhibitory substance (or substances) formed by the h− bacteria (Ryan and Schneider, 1949a). On the other hand, cultures aerated by shaking are limited after adaptation by the exhaustion of glucose; no inhibitory substances are produced nor does the pH drop appreciably (Ryan and Schneider, 1949b). Obviously, under the two conditions different mechanisms are involved in restricting the growth of h+ bacteria. And yet there must be something in common, for the curves describing the levels of adaptive growth under the two conditions are almost identical.

Some of the results secured under aerated conditions required an explanation that assumed that h− bacteria in the stationary stage continue to consume glucose without growing. If this assumption were true, it would be possible to explain the restriction h− bacteria exercise over the adaptive growth of h+ organisms. On a limiting concentration of histidine, h− bacteria will grow until the supply of amino acid is used up. They will then enter the stationary stage and continue to assimilate glucose. In the meantime the h+ bacteria will increase from the very small numbers in which they were originally present. The extent to which they can grow will be determined by how much glucose is wasted by the stationary h− bacteria. This will depend upon the number of h− bacteria present, which, in turn, will depend upon the amount of histidine originally in the culture. Similarly, if in unshaken cultures h− bacteria produce acid and inhibitor(s) in the stationary stage, the same sort of influence on the growth of h+ bacteria could be exerted, although by a different means.

The experiments to be reported were designed to test these hypotheses. The

materials and methods used were the same as those described in the papers already mentioned.

<div align="center">RESULTS</div>

Aerated cultures. In order to determine whether glucose is consumed by h— bacteria in the stationary stage a series of shaken cultures were established, each containing a limiting concentration of 1.6 μg histidine per ml. One of these received an additional lot of 25 μg histidine per ml at the time of inoculation. The
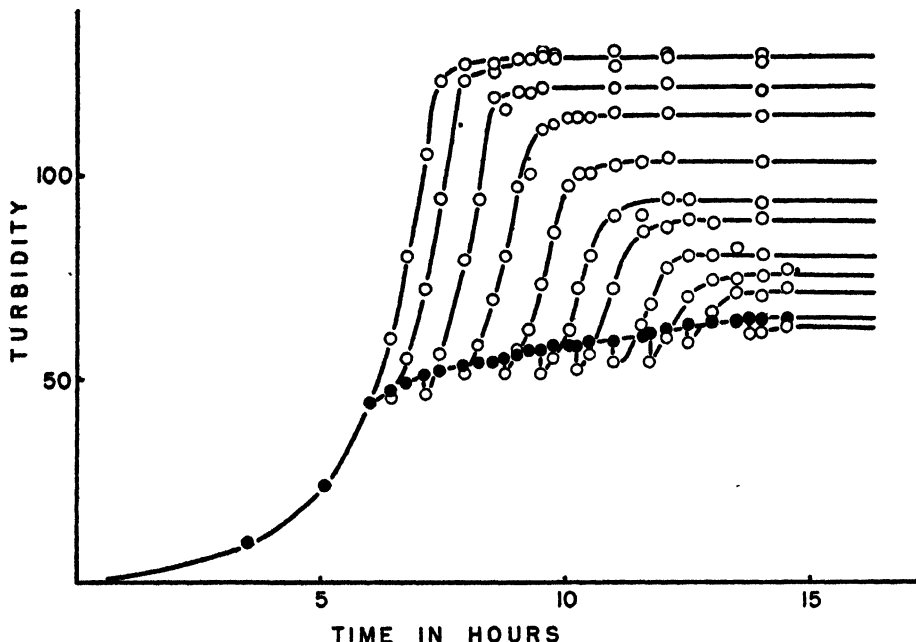
Figure 1. The effect of the addition of optimal amounts of histidine (25 μg per ml) to shaken h— cultures that began growth in the presence of 1.6 μg per ml. The first addition was made at the time of inoculation (0 hours) and the further growth of that culture is described by the curve to the left. The times at which other cultures received their additions are denoted by the slight reductions in turbidity which occurred. The base curve (solid circles) for growth in 1.6 μg histidine per ml is drawn for the average of those cultures that had not yet received their additional histidine.

remainder received similar supplements at various times thereafter. The results of such an experiment are shown in figure 1. The original histidine addition allowed the maximum growth (ca. 130 units) that the glucose in the medium would support. Thereafter the same histidine addition allowed less and less growth until eventually no new growth occurred after the extra histidine was added. At this time glucose was exhausted from the medium, as was shown by the fact that the addition of new glucose resulted in immediate and normal growth. Indeed, after each histidine addition the stationary level of growth achieved was determined by the absence of glucose, as was shown in the same manner.

A plot of the amount of new growth after histidine addition, such as is shown

in figure 2, actually describes the time course of glucose disappearance. If we assume that each unit of growth involves the consumption of the same amount of glucose, then by dividing the total amount of glucose by the total units of growth (5 mg ÷ 130) we secure the glucose equivalent of one turbidity unit. For each interval between histidine additions the decrement in new growth allowed is observed and this multiplied by the glucose equivalent gives the amount of glucose consumed by the stationary h− bacteria during that interval. Knowing the number of h− bacteria present during the interval, we can, by division, de-
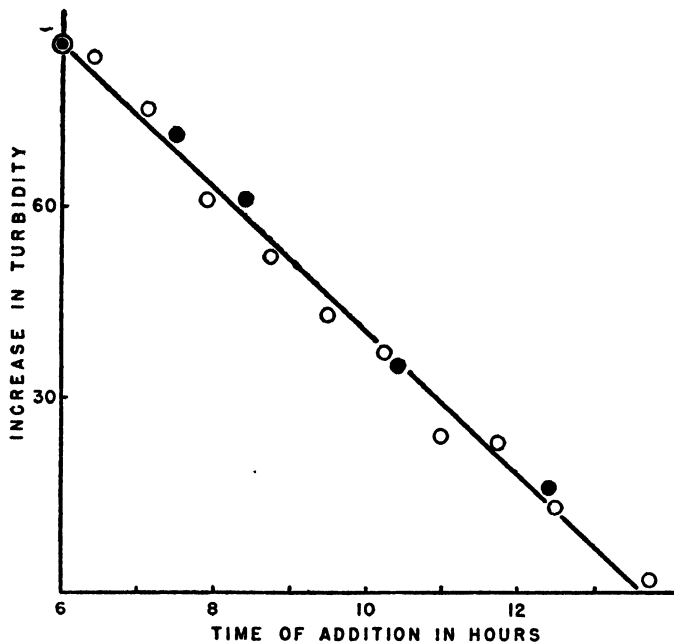
Figure 2. The extents to which new growth occurred upon the addition of 25 μg histidine at different times to shaken h− cultures that began to grow in the presence of 1.6 μg histidine per ml. Six hours is the time at which the cultures began to enter the stationary phase. The points indicated at that time are for the growth of cultures that received their supplement at the time of inoculation. The open circles are for the data shown in figure 1 and the closed circles are for data from a second experiment.

termine the glucose consumed per bacterium. This can be converted to glucose consumed per bacterium per generation time (54 minutes), which is 0.13 × 10^{-9} mg.

It will be seen from figure 1 that during the period in which the glucose is used up the population is not exactly stationary; rather it increases slightly before entering a level state. In order to determine whether the rate of glucose consumption calculated is that of the true stationary stage, an experiment was carried out in which a culture of h− bacteria grown in the presence of 25 μg histidine per ml was washed and resuspended in a series of flasks containing minimal medium. At various times histidine additions were made, and a series of curves similar to those in figure 2 was obtained. These cultures showed a barely perceptible in-

crease during the period in which the glucose was used up, and yet the rate of utilization was again 0.13×10^{-9} mg per bacterium per generation time. Figure 2 was chosen instead for publication because the data it portrays were secured from conditions similar to those under which adaptation occurs.

It is obvious then that the qualitative requirements of the hypothesis have been met by experiment.

Unshaken cultures. In unshaken cultures the situation is somewhat more complicated. Sugar is not exhausted during growth; rather the pH drops and an inhibitory substance (or substances) is increased in concentration. On intermediate concentrations of histidine, where adaptive growth of the h+ bacteria is depressed, the pH is limiting. But these limiting pH's do not prevent the growth of the h+ bacteria when they are by themselves. Rather the pH that will prevent the growth of h+ bacteria depends upon the concentration of inhibitor(s) present. If the inhibitor (or inhibitors) is produced during the stationary stage by h− bacteria, then a means is available of understanding the depression of adaptive growth.

A number of unshaken cultures of h− bacteria in 1.6 μg histidine per ml were allowed to grow and to begin to enter the stationary stage. At this point the pH of one culture was determined, and it was titrated with sterile sodium hydroxide to a pH of 7.0. This culture was then discarded, but the same amount of alkali was added to another culture. This second culture also received histidine to the extent of 25 μg per ml. A third culture received the same amount of histidine but no alkali. At intervals during the stationary stage other cultures in the series received the same treatment. The further growth of these cultures was measured, and a series of curves resembling those in figure 1 was obtained.

Figure 3 summarizes the results, and although there is considerable scattering of points, the interpretation demanded is clear. The lower curve describes the decrease with time in the amount of growth secured after the addition of histidine. After about 16 hours no further growth is obtained. The pH drops during this time in an identical fashion, and at 16 hours is less than 6.1. In other words, the amount of growth obtained by the h− bacteria is a function of the pH at the time new histidine is added. At a pH of 6.1 histidine addition will not reinitiate growth; pH has become limiting in place of histidine. But a pH of 6.1 will allow more than 80 units of growth when h− bacteria are inoculated into 25 μg histidine per ml (Ryan and Schneider, 1949a, figures 4 and 5). Obviously this growth potential has been changed by the activity of the large number (33×10^8) of h− bacteria in the stationary stage. That these bacteria release something into the medium which prevents growth at a pH of 6.1 or less in 25 μg histidine per ml is shown by the upper curve in figure 3, which portrays the extra growth obtained with histidine plus neutralization. When the first neutralization was made at 6¾ hours, just as the h− bacteria were entering the stationary stage, new growth occurred equal to that allowed by the medium (ca. 130 units). Thereafter less and less growth occurred after neutralization and histidine addition until, at about 22 hours, such treatment elicited no growth response. At this time there is sufficient inhibitor (or inhibitors) present to prevent growth even at a pH of

7, for the addition of fresh minimal medium with glucose and histidine and enough alkali to neutralize the culture did not stimulate new growth. The production of inhibitor(s) in this experiment was determined directly by studying the effect of filtrates on the rate of growth of h+ bacteria by the method described in the previous publications (Ryan and Schneider, 1949a,b). A neutralized filtrate secured at 6¾ hours, when the h− culture was just entering the stationary stage, showed a depression of growth of only 5 per cent, which is not significantly different from 0. On the other hand, a neutralized filtrate secured after 33½ hours completely stopped the growth of the h+ bacteria. The slope of the upper curve in figure 3 is actually a measure of the rate of inhibitor
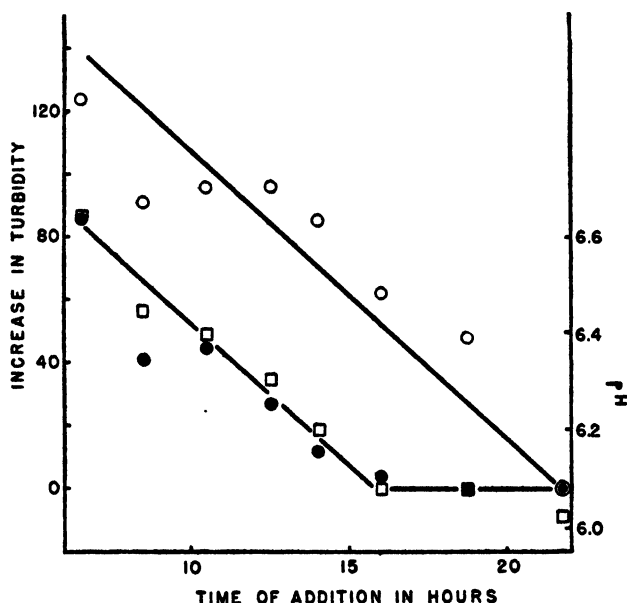
Figure 3. The extents to which new growth occurred upon the addition of 25 μg histidine at different times to unshaken h− cultures which began to grow in the presence of 1.6 μg histidine per ml. The closed circles are for cultures that received only additional histidine. The squares represent the pH's at the time of addition. The open circles are for cultures that, in addition to receiving histidine, were neutralized.

production, and it is the same as the slope of the lower curve. This relationship shows that the amount of growth allowed by the addition of histidine to the stationary h− culture is not simply a matter of pH, but that the amount of growth possible at any pH changes as a function of the concentration of inhibitor(s) present.

Similar curves were obtained for the reinitiation of growth in h− cultures in the stationary stage on 0.8 μg histidine per ml. These showed less scatter than is observed in figure 3 and differed only in possessing lesser slopes. The lower curve extinguished at 26 hours and the upper curve at 36 hours. This was to be expected because of the smaller-size of the h− population (23 × 10⁸) in cultures grown on 0.8 μg histidine per ml.

It can be concluded that in unshaken h− cultures grown on limiting concen-

trations of histidine the exhaustion of that amino acid restricts growth and the population enters the stationary stage. Through the continued production of acid by the h— bacteria the pH next becomes limiting. Eventually the concentration of an inhibitor (or inhibitors) becomes so great that new growth is no longer possible.

While this is happening, h+ back mutants in the h— culture are able to grow only until the pH becomes limiting; for they, like the h— bacteria, have acid tolerances determined by the amount of inhibitor(s) present (Ryan and Schneider, 1949a). On low concentrations of histidine, where there are small populations of h— bacteria, the pH drops slowly and there is a slow increase in inhibitor concentration. Here the adaptive growth of the h+ bacteria is greater than in high concentrations of histidine. In 1.6 μg per ml, for example, the pH becomes limiting in about 16 hours, long before the h+ back mutants have had a chance to grow to visible turbidity and express themselves as adaptation.

This hypothesis even explains the timing with which unshaken cultures adapt. The lower the level of adaptive growth the later and slower does it occur (Ryan and Schneider, 1948, figure 2). This is undoubtedly due to the fact that where adaptive growth is poor there the pH and inhibitor concentration are such as to slow down the rate of growth of h+ bacteria. Because of this slow rate the h+ bacteria reach the level of visible turbidity later despite the fact that their numbers may be added to by back mutation in the large h— population. This is the reverse of the situation in shaken cultures in which adaptive growth of h+ bacteria is always at the same rate (Ryan and Schneider, 1949b, figure 3). Consequently, adaptation there occurs earlier in the larger h— populations because of back mutation, although glucose is used up faster and the level of adaptive growth is less.

Quantitative verification of the hypothesis. Further quantitative study of unshaken cultures was abandoned because of our inability to measure the inhibitor(s) with precision. However, the utilization of glucose by aerated cultures in the stationary phase was determined accurately and found to be 0.13×10^{-9} mg per bacterium per generation time. The rate of utilization of glucose during growth can also be calculated in the following way: During growth to a turbidity of 130 about 6.5×10^9 bacteria are produced. This requires the consumption of 5 mg of glucose, which when divided by 6.5×10^9 gives 0.77×10^{-9} mg per bacterium. Since one bacterium is produced from another per generation, the glucose consumed per bacterium per generation during growth is 0.77×10^{-9} mg. This rate is the same for growing h+ and h— bacteria.

With this knowledge it is possible to calculate the amount and composition of growth on any histidine concentration for any mixture of h— and h+ bacteria introduced as an inoculum. A running tabulation is kept of the amount of sugar consumed by the h— bacteria as they grow generation by generation from the known number in the inoculum to the number the histidine in the medium will support. During this period a similar tabulation is kept of the increase in number of the h+ bacteria (which have the same generation time) and of the sugar they consume. Thereafter for every generation time the h— consume glucose at the

stationary rate while the h+ continue to grow and consume glucose at the higher rate of growing bacteria. Eventually the sum of the glucose consumed will equal 5 mg, and this occurs at the end of growth. The total number of h− and h+ bacteria is thus calculated and this figure divided by a factor of 5 × 10⁶ bacteria per turbidity unit yields the final turbidity of the culture. Corrections are also made for the production of new h+ back mutants by the h− bacteria during growth using the previously determined rate of 5×10^{-8} per h− bacterium per generation (Ryan, 1948).

Table 1 shows a comparison of the predicted and observed growth of various mixtures of h+ and h− bacteria in the absence of and in the presence of a limiting amount of histidine. The comparison is good and verifies the facts and

TABLE 1

Comparison of the extent of growth, in turbidity units, calculated and observed for mixtures of h+ and h−

	INOCULUM		TURBIDITY	
	Number of h−	Number of h+	Calculated	Observed
In medium devoid of histidine	0.75×10^8	1.0×10^7	109	111
	7.5×10^8	1.0×10^7	90	88
	15×10^8	1.0×10^7	70	65
	38×10^8	1.0×10^7	32	26
	75×10^8	1.0×10^7	0	6
	94×10^8	1.0×10^7	0	3
	7.5×10^8	1.0×10^6	86	85
	0.75×10^8	1.0×10^5	89	108
	1.4×10^8	200	103	109
In 1.6 μg histidine per ml	1.4×10^6	0	49	49
	1.4×10^6	1.0×10^2	50	62
	1.4×10^6	1.0×10^4	67	75
	1.4×10^6	1.0×10^6	100	104
	0	1.0×10^6	111	112

assumptions used in calculating the rates of glucose utilization. The correspondence also lends credence to the hypothesis that the glucose consumed without attendant growth by stationary h− bacteria determines the level to which adaptive growth can occur.

Further verification was secured by a comparison of the predicted and observed levels of adaptive growth and the predicted and observed composition of cultures adapted on different concentrations of histidine. The h− inoculum was determined by plating to contain 3.25×10^7 h− bacteria and 5 h+ bacteria, and the calculated and observed levels of adaptive growth are shown in figure 4; table 2 shows the calculated and observed composition of these adapted cultures. Once again the comparison is satisfying. The few observed discrepancies may not be surprising in view of the assumptions made in these calculations. For example, the generation time was not measured in this experiment but was allowed to

equal the average of previous experiments; it was assumed that there was no lag period although actually there was, if lasting only an hour or so; it was
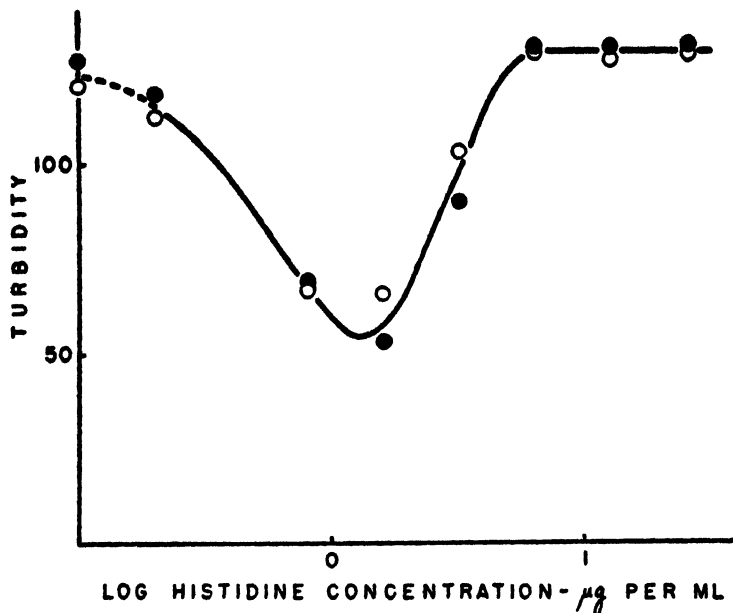
Figure 4. The levels of adaptive growth of h— cultures on different concentrations of histidine 34 hours after inoculation. The open circles are for observed data and the closed circles are for values calculated according to the hypothesis described in the text. The dotted lines go to values determined in the absence of histidine.

TABLE 2

Calculated and observed values for the levels of adaptive growth achieved on different concentrations of histidine

(Inoculum contained 3.25 × 10^{7} h— and 5 h+ bacteria)

HISTIDINE CONCENTRATION	NUMBER OF H—		NUMBER OF H+	
	Calculated	Observed	Calculated	Observed
μg per ml				
0	0.03 × 10⁹	<0.1 × 10⁹	6.4 × 10⁹	6.1 × 10⁹
0.2	0.13 × 10⁹	<0.1 × 10⁹	5.8 × 10⁹	5.3 × 10⁹
0.8	0.78 × 10⁹	0.75 × 10⁹	2.7 × 10⁹	2.0 × 10⁹
1.6	2.7 × 10⁹	1.6 × 10⁹	1.6 × 10⁵	2.1 × 10⁵
3.2	4.5 × 10⁹	4.4 × 10⁹	5.1 × 10³	9.8 × 10⁴
6.4	6.5 × 10⁹	6.8 × 10⁹	1.0 × 10³	1.2 × 10³
12.8	6.5 × 10⁹	5.9 × 10⁹	1.0 × 10³	0.9 × 10³
25.6	6.5 × 10⁹	6.9 × 10⁹	1.0 × 10³	0.7 × 10³

assumed that no lysis occurred or that there was no use made of any other carbon and energy source than the glucose supplied; it was assumed that the number of h+ bacteria introduced with each inoculum was 5 although this was the average number determined by plating and the distribution of numbers of

h+ bacteria would follow the equation of Poisson; and, finally, it was assumed that mutations from h— to h+ did not occur in the stationary phase when the h+ were selected for. Despite the uncertain nature of these assumptions it seems fairly evident that the proposed hypothesis can quite adequately account for the facts of adaptation.

<div align="center">DISCUSSION</div>

The ability of microorganisms to adapt to dispensing with growth factor requirements has long been known (Ryan, 1948). Indeed, the phenomenon had been called "training" and was given the interpretation of the induction of an inherited modification by the environment. However, the recent development of the science of microbiological genetics has yielded reasons for reconsidering such interpretations. Fildes and Whitaker (1948), for example, approached, as a problem in population ecology, the question of "training" *Eberthella typhosa* to dispense with a requirement for tryptophan and have reversed their position. Tryptophan-requiring strains were shown to contain tryptophan-independent mutants with a small frequency, and the latter accounted for the adaptive growth in the presence of low concentrations of tryptophan. This study can be contrasted with that of Peacock and Hinshelwood (1948), who conclude that adaptation to dispensing with amino acids for growth is a matter of autosynthetic enzyme systems and is not the selection of mutants. They go so far as to state that "the restoration of this ability [to utilize ammonia] cannot be attributed to natural selection, since the damaged strain originally arose from one irradiated cell." This statement is, of course, made in ignorance of the fact that, in a culture arising from a single cell and containing about 10^{10} bacteria, if a mutation occurs even at a rate as small as 10^{-8} per bacterium per generation, hundreds of mutants will be present at the end of growth. The main contribution that genetics makes to the problem of adaptation in microorganisms is to focus attention on the fact that a bacterial culture is not like a solution of enzymes but is rather a *population*, each member of which has a small but definite chance of mutating to a given condition in a nonadaptive way. It is through the action of selection that changes in mutant frequencies within the culture are brought about.

Selection may act in many ways through a variety of mechanisms. It may be dynamic in the sense that one strain will grow faster than another and as a consequence will increase in proportion. Roepke et al. (1944) have presented evidence that some growth-factor-requiring mutants of *Escherichia coli* grow faster than the normal type. However, the fastest, in three determinations, grew less than 5 per cent faster than normal, a difference that may not be significant. Monod (1946), on the other hand, showed that a methionine-requiring strain of *Escherichia coli*, when supplied with methionine, grew significantly faster than normal. It is still necessary to bear in mind the high probability that rates determined on strains growing separately may not reflect those occurring in mixtures (Ryan, 1946). Experiments are at present being conducted in our laboratory on the question of such selection in mixtures of h— and h+ bacteria and on the kinetics of population equilibrium (Ryan and Schneider, 1949d).

Selection may also act at the end of growth. If both components have ceased to grow, a shift in proportion may occur through differential viability (Braun, 1947; Witkin, 1947). Or in the absence of such a differential, if one of the components ceases growth before the other, the final population will differ from the inoculum. This is the situation existing in populations of h— bacteria grown on limiting amounts of histidine. In the presence of high concentrations of histidine some other factor, such as exhaustion of glucose in aerated cultures or the production of acid and inhibitor(s) by unshaken cultures, becomes limiting. These factors have equal influences on h+ and h— individuals, and as a result both components slow down and stop their growth simultaneously. Since, in addition, under the conditions examined there is no differential growth rate or viability, the proportion of h+ and h— remains unmodified and there is no selection. On the other hand, on limiting concentrations of histidine the growth of the h— component of the population ceases when the amino acid is exhausted. The h+ component continues to grow. The extent to which this growth takes place depends upon the activity of the h— bacteria in the stationary phase. In aerated cultures the critical activity is the consumption of glucose; in unshaken cultures it is the continued production of acid and of inhibitor(s) which determines the extent of growth at any pH. The more h— bacteria in the stationary phase, the sooner the medium will not support growth and the smaller will be the final population of h+ organisms. The amount of histidine determines the size of the stationary population of h— bacteria, and it is because of this relationship that adaptation occurs in different degrees on different concentrations of histidine. Thus, by different means (the production of acid and inhibitor(s) or the exhaustion of glucose) through a common denominator (modification of the medium by bacteria in the stationary phase) a similar behavior, the depression in the adaptation curve, can be achieved.

The fact that the growth curve of a culture that adapts has two growing and two stationary phases is reminiscent of the phenomenon of diauxie described by Monod (1942). Whenever curves of this sort are obtained, it is necessary by analysis of the genetic composition of the population to distinguish between the step-wise activity of different enzyme systems and the selection of mutants.

The cause of cessation of growth in bacterial cultures has been subject to much investigation. Usually these studies were carried out on complex media (Coblentz and Levine, 1947). By the use of chemically defined media it is possible to set one or another factor limiting; but even then, in the closed system of a bacterial culture, other factors that may not be critical at the moment also change. For example, histidine can be set limiting for histidineless bacteria. In unshaken cultures with 0.5 per cent glucose, even before the amino acid is used up, the pH drops enough to gradually decrease the rate of growth. As a result, in the range of visible turbidity the rate of growth is not logarithmic. In shaken cultures with 0.05 per cent glucose, the same bacterial populations are obtained without a decrease in pH and at a logarithmic rate of growth. But even here, although lack of histidine may stop growth, the exhaustion of glucose will eventually prevent further growth when new histidine is added. In a closed system the

limiting conditions are bound to change. The more complex these changes are, the more variables are involved and the more unlikely it becomes that consistent results can be obtained. This probably accounts for the nature of our results in unshaken cultures.

At this point it may be well to ask whether the sort of growth restriction observed with h+ and h— bacteria is a special or a general case. Actually there can be little doubt of its general nature. Microcolonies of h— bacteria on minimal agar plates have been shown to give rise to adapted macrocolonies that consist of h+ bacteria (Ryan and Schneider, 1949c). The chance that an h+ back mutant will form an adapted colony is influenced by the h— bacteria in the microcolony where it arises and by the total number of microcolonies on the plate. Newcombe (private communication) has found that phage-sensitive *Escherichia coli* may, when crowded on the surface of an agar plate, suppress the growth of recently formed phage-resistant mutants. Zamenhof (1946) has claimed that citrate-fermenting mutants of *Escherichia coli* are specifically inhibited by some unknown product of the metabolism of the parent non-citrate-fermenting bacteria. Further, Guthrie (1949) finds that the frequency of reversion of cultures of a purine-requiring mutant of *Escherichia coli* is greater in low concentrations of purine where there are fewer parent organisms.

In their studies of a tryptophan-independent mutant of *Lactobacillus arabinosus* Wright and Skeggs (1945) found that increasing concentrations of that amino acid first depressed and then allowed its rapid growth. We have confirmed this finding but as yet have not been able to discover why the tryptophan-*independent* mutant, when it is presumably growing alone, shows this depression. However, the close similarity between figure 3 of Wright and Skeggs's paper and our curves for the adaptation of h— cultures leads us to suspect that a common mechanism is involved. Similarly, in the case of the selection of leucineless, in preference to leucine-independent, back-mutant nuclei in heterocaryons of *Neurospora crassa*, identical curves were obtained (Ryan, 1946, figure 5). In some way, as yet not understood, leucineless nuclei were able to limit and even prevent the adaptive growth of leucine-independent nuclei. The contention of Giles and Lederberg (1948) that selection against inositol-independent back mutant nuclei does not occur is unfounded. They argue that, since the frequency of reversions seems to increase with the concentration of inositol in the medium, there can be no selection for inositolless nuclei. However, since the mycelial mass, and hence the number of inositolless nuclei, increases with inositol concentration, one would expect more back mutations to occur on high concentrations. It would be necessary to show that the frequency of reversions increased *in proportion* to nuclear number. A χ^2 test of their data indicates that there is no evidence that any increase in frequency of reversions occurs with increasing inositol concentration. Hence, even the factual basis of their argument is unsound. Experiments involving the artificial mixture of inositolless and inositol-independent nuclei, such as were performed in the leucineless study, would be advisable before conclusions are drawn for this case. Finally, mention should be made of the discovery by Fries (1946) that reversion in *Ophiostoma multian-*

nulatum from a requirement for reduced sulfur to an ability to use sulfate occurs more frequently when no reduced sulfur is present. This phenomenon appeared to Freis to be an example of the induction of an adaptive mutation by the absence of reduced sulfur. More likely the phenomenon was once again the suppression of the mutant by the parent form.

Cases like these appear, then, to be commonly found among microorganisms. Parent organisms may regulate the expression of a mutation in a way conditioned by the environment. Without the proper recognition of the population mechanics involved, such cases are easily misinterpreted as "Lamarckian" phenomena. Careful analysis has in many cases shown, however, that it is not the mutation, or change in the individual, which is adaptive (Ryan, 1948). Rather it is the interaction of the various components that changes the population in an adaptive way (Darwin, 1859).

SUMMARY

The restriction of the growth of histidine-independent bacteria by histidineless organisms was found, in unshaken and in aerated cultures, to be due to different causes. Yet in both instances there is the common principle that involves the modification of the medium by histidineless bacteria in the stationary phase.

In aerated cultures, histidineless bacteria cease to grow when the histidine supply of the medium is exhausted, but they continue in the stationary stage to consume glucose until it too is absent from the medium. In the meantime the small number of histidine-independent back mutants contained by histidineless cultures continue to grow. They are not influenced by the exhaustion of histidine from the medium but cease growth when the glucose is gone. The time of glucose disappearance, and hence the extent to which histidine-independent bacteria can undergo adaptive growth, depends upon the number of histidineless bacteria that are consuming glucose in the stationary stage. The number of histidineless bacteria is, in turn, a function of the initial concentration of histidine. For this reason, the adaptive growth of histidineless bacteria is greatest on low concentrations of histidine and eventually does not take place as the histidine concentration becomes high enough.

The rates of glucose utilization during growth and in the stationary stage have been determined and used to calculate the expected extents of adaptive growth and the expected composition of adapted cultures. The close correspondence of theory and experiment verifies the hypothesis.

In unshaken cultures the adaptive growth of histidine-independent bacteria is regulated in a similar fashion. The glucose supply, however, does not become limiting. Rather, the histidineless bacteria in the stationary phase produce acid and an inhibitor (or inhibitors), which limit the amount of growth that is possible at any pH. This modification of the medium also occurs as a function of the number of histidineless bacteria and is responsible for the differences in the restriction of the adaptive growth of histidine-independent bacteria on different concentrations of histidine.

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STUDIES ON THE ASPARTIC ACID DECARBOXYLASE OF RHIZOBIUM TRIFOLII

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Virtanen and Laine (1937) were the first to suggest the existence of an aspartic acid decarboxylase in *Rhizobium trifolii*, by the isolation of *beta*-alanine from a 47-day fermentation mash containing aspartic acid. Shive and Macow (1946), working with hydroxyaspartic acid as a competitive analogue for aspartic acid in the metabolism of *Escherichia coli*, found that the addition of this compound to the growth medium inhibited the proliferation of the organism and that this inhibition was completely prevented by the addition of aspartic acid, pantothenic acid, or *beta*-alanine. They concluded from these results that aspartic acid was the precursor of *beta*-alanine, which in turn becomes part of the pantothenic acid molecule.

It has been reported recently (Mardeshev *et al.*, 1948, 1949) by several Russian workers that they have found an organism, *Pseudomycobacterium*, that is capable of decarboxylating aspartic acid, as measured by CO_2 production in a Warburg vessel. They further state that the enzyme has been prepared in a cell-free state (Mardeshev *et al.*, 1948) and that the coenzyme of the decarboxylase has been found to be pyridoxine phosphate (Mardeshev *et al.*, 1949).

It has more recently been reported from this laboratory (Billen and Lichstein, 1949) that washed cell suspensions of *Rhizobium trifolii* contain an aspartic acid decarboxylase as determined by microbiological analysis for the end product of the decarboxylation, *beta*-alanine. Because of the importance of such an enzyme, further studies have been made and the data are herewith presented.

EXPERIMENTAL METHODS

Preparation of active cell suspensions. The organism employed in most of the work was a strain of *R. trifolii* carried as a departmental stock culture. This organism, as well as others to be reported later, was grown in a medium that consisted of the following: 10 grams each of yeast extract, tryptone, and glucose; 1 gram of DL-aspartic acid; and 1,000 ml of distilled water.

A 1-ml inoculum of a 24-hour culture of *R. trifolii* was introduced into 200 ml of the growth medium. The flask was then incubated on a mechanical shaker at room temperature, which fluctuated between 24 and 30 C. The mechanical shaker provided a convenient means of aeration and made possible the harvest of large quantities of cells. After 16 to 18 hours' incubation (final pH 4.5 to 5.5), the cells were harvested by centrifugation, suspended in distilled water equal in volume to the growth medium, and recentrifuged. This washing was repeated twice to remove as much endogenous material as possible, and the washed cells were re-

suspended in distilled water. The concentration was determined in terms of bacterial nitrogen per ml of suspension by measuring turbidity in a Klett-Summerson photoelectric colorimeter and converting into terms of nitrogen content by the use of a previously standardized table.

Experimental procedure. In general an experiment was run in the following manner: A series of pyrex tubes (13 by 100 mm) was placed in a metal rack. To each tube in the series was added 1 ml of 0.5 M phosphate buffer of the desired pH, 0.1 ml of 0.1 M L-aspartic acid, and finally the cell suspension. This was then brought to a constant volume of 2 ml by the addition of distilled water and the tubes were placed in a water bath at the desired temperature. The reaction was allowed to proceed for a specific time and then stopped by immersing the tubes in boiling water for 5 minutes. Controls were run in the same manner without added
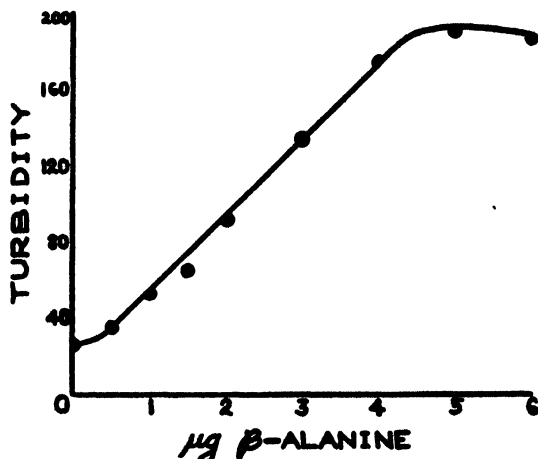
Figure 1. Response of *Saccharomyces fragilis* to *beta*-alanine (modified Snell medium; 24 hours; 30 C).

aspartic acid. The tubes were then centrifuged and aliquots of the supernatant assayed for *beta*-alanine.

Assay for beta-alanine. The aspartic acid decarboxylase activity of the washed cells was determined by microbiological assay for *beta*-alanine in the supernatant. A synthetic medium described by Snell *et al.* (1940) was employed first. The response by the assay organism was found to be limited to a narrow range of approximately 0.1 to 0.2 μg of *beta*-alanine in this medium. The addition of nicotinic acid (100 μg), *para*-aminobenzoic acid (20 μg), and 50 ml of a 10 per cent vitamin-free acid-hydrolyzed casein to 950 ml of the assay medium, followed by adjustment to pH 5.5, increased the range of assayable *beta*-alanine 30- to 40-fold (figure 1).

The organism employed for the assay was *Saccharomyces fragilis* (ATCC 2360). This organism was grown for 24 hours in a medium composed of 1 per cent each of yeast extract, tryptone, and glucose; harvested by centrifugation; washed twice with distilled water; and resuspended in distilled water. The assay medium was inoculated with washed cells until a faint turbidity was noted. Five ml of

inoculated assay medium were added to aliquots of the supernatant made to a volume of 2 ml with distilled water that had been steamed in an autoclave at 2 to 5 pounds steam pressure for 10 minutes and cooled. The assays were made in pyrex glass tubes (24 by 200 mm) with cotton plugs inserted before steaming to avoid condensation of fluid into their contents. The tubes were then incubated at 30 C and turbidity measurements made after 20 to 24 hours in a Klett-Summerson photoelectric colorimeter. The concentration of *beta*-alanine in the supernatants was calculated from standard curves included in each experiment.

$$Q_{(N)} \beta\text{-ALANINE} = \frac{\mu g\ \beta\text{-ALANINE}}{\text{TIME (HR)} \times \text{MG N}}$$
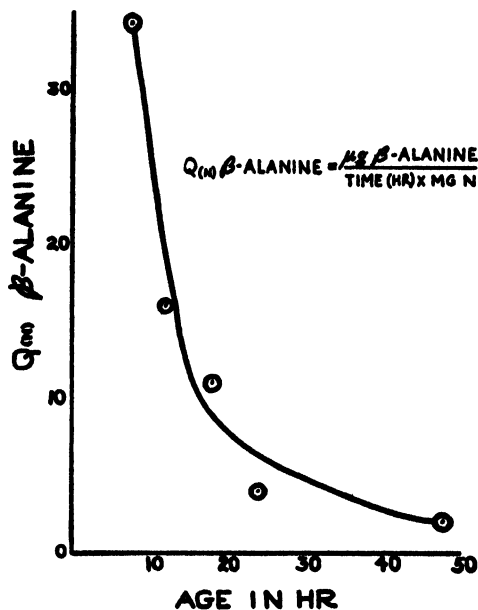
Figure 2. Effect of age of culture on enzyme activity (30 minutes; 30 C; 0.5 M phosphate buffer, pH 6).

EXPERIMENTAL RESULTS

The results of several experiments indicated little effect of temperature on enzyme production when the organisms were grown between 22 to 38 C. The $Q_{(N)\ beta\text{-alanine}}$[1] for several temperatures was 5.5 at 22 C, 6.5 at 30 C, and 6.4 at 38 C.

The age of the culture had marked effect on enzyme activity. It was found that the activity was greatest during the logarithmic phase (8 to 12 hours) and decreased rapidly with time (figure 2). In this experiment 1 ml of a 12-hour culture, aerated on the mechanical shaker, was used as the inoculum in order to obtain significant growth from the 8- and 12-hour cultures.

An experiment was made using 0.5 M phosphate buffer of varying pH values from 3 to 9 to determine the effect of hydrogen ion concentration on enzymatic activity. The final pH of each tube was determined after the cell suspension had been added. The reaction was stopped by boiling, and the pH of the supernatant

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[1] $Q_{(N)beta\text{-alanine}}$ = μg *beta*-alanine produced per mg bacteria nitrogen per hour.

was adjusted by the addition of 1 ml of 1 M phthalate buffer (pH 5) in order to equalize the pH of all the supernatants, thus preventing possible adverse effects of widely varying pH's on the results of the assay. The enzyme shows optimal activity between pH 5.2 and 6.2, with activity falling off sharply on either side of this range (figure 3). This is in agreement with the general range of optimal pH for the other known amino acid decarboxylases, which vary between 2.5 and 6.0 (Gale, 1946).

The optimal temperature of reaction is approximately 46 C, the increment of activity with temperature being at a maximum at this point (figure 4). It will
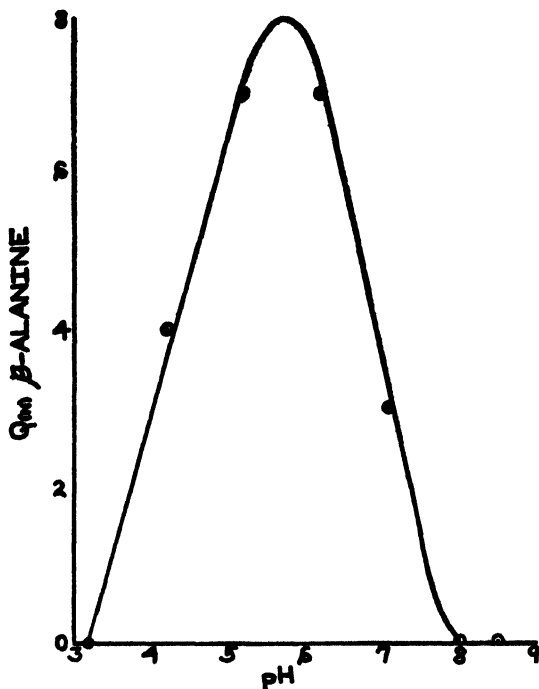
Figure 3. Effect of pH on enzyme activity (120 minutes; 35 C; 0.65 mg bacterial nitrogen per tube; 0.5 M phosphate buffer).

be noted that the optimal temperature is considerably higher than that of growth, namely, 30 C. The probability exists that the enzyme deteriorates rather rapidly at the higher temperatures (54 and 46 C) and that over a longer period of time the total decarboxylation would be greater if incubated at a lower temperature.

An experiment was performed to determine whether aspartic acid decarboxylation goes to completion as determined by complete conversion to *beta*-alanine. One series of tubes contained a relatively large concentration of substrate (1,330 μg) and another series of tubes contained a limited concentration (66 μg). It was found that the reaction proceeds normally for 5 hours in the presence of large amounts of substrate, whereas in the tubes containing the smaller concentration, the activity stops after 2 hours (figure 5) and the rate of decarboxylation is con-

Figure 4. Effect of temperature on enzyme activity (1.4 mg bacterial nitrogen per tube; 0.5 M phosphate buffer, pH 5.5).

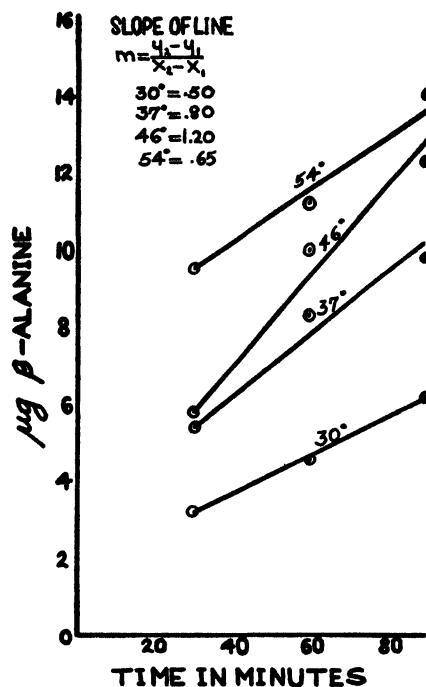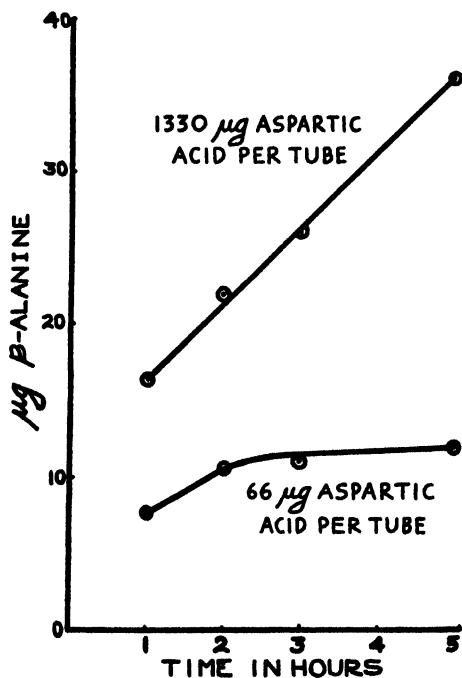Figure 5. Effect of substrate concentration on enzyme activity (0.5 mg bacterial nitrogen per tube; 36 C; 0.5 M phosphate buffer, pH 5).

siderably lower than in the former case. It is evident from this that the rate of activity is dependent on substrate concentration and is probably influenced by the law of mass action over a certain range of substrate-enzyme concentration. It is also to be noted that only 15 to 20 per cent of the substrate can be accounted for as *beta*-alanine when the reaction is stopped after 2 hours. A possible explanation for this lies in the competition for the substrate by other enzyme systems. These include decarboxylation to *alpha*-alanine by a change in the site of decarboxylation, deamination to fumaric acid, and transamination. This problem is under investigation in our laboratory at the present time. Preliminary data suggest that this organism contains an aspartic acid deaminase that is active at pH 5.5.

Several other organisms were tested for their decarboxylase activity. The only other organism studied that showed activity was *Escherichia coli* (Texas). A $Q_{(N)}$ *beta-alanine* of 1 was obtained using cells from a 22-hour culture. This is approximately half of the activity of *R. trifolii* under similar conditions. *Lactobacillus bulgaricus*, *Streptococcus faecalis* R, *Corynebacterium diphtheriae*, *Saccharomyces fragilis*, *Micrococcus pyogenes* var. *albus*, and *Aerobacter aerogenes* gave negative results.

DISCUSSION

The results of these experiments provide evidence for the presence of an enzymatic aspartic acid decarboxylase system in *R. trifolii* and *E. coli* (Texas), although in small quantity. The importance of such an enzyme system can readily be appreciated when it is realized that *beta*-alanine is part of the pantothenic acid molecule. If aspartic acid decarboxylase is concerned with the supply of adequate amounts of *beta*-alanine for the synthesis of pantothenic acid, the low activity found should not be surprising as pantothenic acid is needed in relatively small amounts by those organisms that require it for growth.

The low activity might also present an explanation for the negative results obtained in our laboratory when CO_2 production was employed as a measure of activity. Assuming, from the results of the work reported, that approximately 20 to 30 μg of *beta*-alanine would be produced in a reaction under optimal conditions over a period of 2 hours, by calculation it would mean that 10 to 15 μg or 5 to 8 μl of CO_2 would be produced. This quantity, produced over a period of 2 hours, would not be detectable by the conventional manometric measurements.

SUMMARY

A modified medium for the microbiological assay of *beta*-alanine is described.

Rhizobium trifolii was found to contain an enzyme system capable of decarboxylating aspartic acid to *beta*-alanine, as determined by microbiological assay for this compound.

A study of the properties of the system was undertaken with the following results: (1) The age of the culture markedly effected enzyme activity, the greatest activity occurring during the logarithmic phase of growth. (2) No marked effect of growth temperature between 22 and 38 C was noted on enzyme activity. (3)

The optimum temperature of activity of washed cells appeared to be 46 C. The calculated slope of *beta*-alanine production versus time was greatest at this temperature. (4) The enzyme shows a pH optimum between pH 5.2 and 6.2 in phosphate buffer, with the activity falling off sharply on either side of this range. (5) The production of *beta*-alanine is markedly influenced by substrate concentration.

The enzyme was found to be present in *Escherichia coli* (Texas) and *Rhizobium trifolii*, but absent under the conditions of the experiments in *Lactobacillus bulgaricus*, *Streptococcus faecalis* R, *Corynebacterium diphtheriae*, *Saccharomyces fragilis*, *Micrococcus pyogenes* var. *albus*, and *Aerobacter aerogenes*.

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GROWTH INHIBITION OF TUBERCLE BACILLI BY ANALOGUES OF PHENYLALANINE

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Many studies have been devoted to the inhibitory action of various metabolite analogues on microbial growth. In most of this work, however, such nonpathogens as the yeasts, lactobacilli, and *Escherichia coli* have served as the test organisms and there has been little attempt to correlate or extend the studies to pathogenic bacteria, especially those organisms synthesizing their own growth factors.

In a recent study we have shown the results obtained when 96 metabolite antagonists were tested on two virulent strains of the tubercle bacillus, an organism capable of synthesizing its own vitamins (Pope and Smith, 1949). Analogues of biotin and p-aminobenzoic acid were found to exert a specific bacteriostatic action on the organism, and Drea (1948) has found that an analogue of phenylalanine, β-2-thienylalanine, is inhibitory for the tubercle bacillus at high concentrations. With such findings as evidence that an inhibition of synthesis or utilization of a metabolite may be produced in a pathogenic organism that has the inherent ability to synthesize its own vitamins and amino acids from simple substances contained in the culture medium, further studies of this kind are indicated.

The present paper reports the inhibition of growth of the tubercle bacillus by five analogues of phenylalanine and the effect of phenylalanine on this inhibition. The compounds studied were β-2-thienylalanine, β-3-thienylalanine, β-2-furylalanine, α-amino-phenylmethane sulfonic acid, and β-hydroxyphenylalanine.

MATERIALS AND METHODS

A virulent human strain of *Mycobacterium tuberculosis*, H37Rv, was used exclusively in these studies. Stock cultures of the organism were maintained on Proskauer-Beck synthetic medium, which also served as substrate in the test cultures. Twenty-ml aliquots containing serial dilutions of the drug under study were inoculated with one loopful of organisms from an actively growing culture. Duplicate tests were run at each concentration level, and four control cultures always were included with each series. Repeat tests were made on all compounds showing any degree of inhibitory action at levels up to 100 mg per cent. After establishment of the minimal effective concentration (MEC) of the drug required to prevent surface growth of the organism completely, phenylalanine was tested for its ability to neutralize the bacteriostatic effect, and the antibacterial index was determined. As used here the antibacterial index is expressed as $\dfrac{C_I}{C_M}$ in which C_I signifies the minimal effective molar concentration of analogue required to

inhibit surface growth of the organism completely, and C_M is the molar concentration of related metabolite required to neutralize the inhibitory effect.

The following amino acids also were tested for reversing activity: alanine, arginine, aspartic acid, cystine, glutamic acid, histidine, isoleucine, leucine, norleucine, lysine, methionine, proline, serine, tryptophan, tyrosine, and valine.

Because of the nature of the growth of the tubercle bacillus as a pellicle on the surface of the medium, growth was graded after a 6-week growth period in terms of $\pm$, $+$, $++$, $+++$, and $++++$. All cultures were coded and readings made by the same investigator.

The five analogues of phenylalanine tested by the foregoing procedure include β-2-thienylalanine, β-3-thienylalanine, β-2-furylalanine, α-amino-phenylmethane sulfonic acid, and β-hydroxyphenylalanine.

TABLE 1

Growth of the H37Rv tubercle bacillus in the presence of phenylalanine analogues

CONCENTRATION OF ANALOGUE	β-3-T	β-2-T	β-2-FA	α-APSA	β-HP
mg %					
0	$++++$	$++++$	$++++$	$++++$	$++++$
0.1	$++++$	$++++$	$++++$	$++++$	$++++$
0.25	$+++$	$++++$	$++++$	$++++$	$++++$
0.5	$++$	$++++$	$++++$	$++++$	$++++$
1	$+$	$++++$	$++++$	$++++$	$++++$
5	0	$++++$	$++++$	$++$	$++++$
10	0	$+++$	$++++$	$+$	$++++$
25	0	$++$	$++++$	0	$++++$
50	0	$\pm$	$++++$	0	$++++$
75	0	0	$+++$	0	$++++$
100	0	0	$\pm$	0	$++++$
Inhibition neutralized by phenyl-alanine	$+$	$+$	$+$	$-$	

β-3-T = β-3-thienylalanine; β-2-T = β-2-thienylalanine; β-2-FA = β-2-furyl-DL-alanine; α-APSA = α-amino-phenylmethane sulfonic acid; β-HP = β-hydroxyphenylalanine.

Additional tests were made with β-3-thienylalanine using as substrate Dubos' sorbitan monooleate medium without albumin. The medium also served as the diluent in preparing serial dilutions of the drug and phenylalanine. The inoculum consisted of 0.1 ml of a homogeneous 7-day culture of H37Rv grown in the basic sorbitan monooleate medium. Tests were conducted in 18-mm matched test tubes containing a total volume of 10 ml. Each culture tube was shaken daily to resuspend the organisms, which tended to settle out, and to break up the small clumps that formed. A homogeneous suspension of organisms was thus obtained, and the extent of growth was determined daily by turbidity measurements on the "lumetron" photocolorimeter.

RESULTS

Table 1 shows the magnitude of inhibition of the H37Rv tubercle bacillus by the different analogues of phenylalanine. Four of the compounds inhibited growth

of the bacillus, but in only three of the cases, β-3-thienylalanine, β-2-thienyl-alanine, and β-2-furylalanine, could the inhibition be overcome by phenylalanine. β-3-Thienylalanine was by far the most effective of the analogues tested, 5 mg per cent preventing completely surface growth. With β-hydroxyphenylalanine at concentrations up to 100 mg per cent no inhibitory activity was observed.

Table 2 presents data on the neutralizing effect of varying concentrations of phenylalanine on the bacteriostasis produced by three of the analogues at their

TABLE 2

Growth of tubercle bacilli in the presence of the minimal effective concentration of analogues and phenylalanine

ANALOGUE	MG % PHENYLALANINE				ANTIBACTE-RIAL INDEX $\left(\frac{C_I}{C_M}\right)$
5 mg %	1	2.5	3.75	5	
β-3-T	+	++	+++	++++	0.96
75 mg %	5	10	20		
β-2-T	+	++	++++		3.62
100 mg %	25	50	75		
β-2-FA.	+	+++	++++		1.42

Figure 1. Effect of β-3-thienylalanine on the growth of H37Rv tubercle bacilli in Dubos' medium.

MEC. β-3-Thienylalanine was found to be not only the most effective inhibitor on the basis of the low concentrations that prevented growth of the organism but also because of the low antibacterial index. This drug was found to have an index of 1.0 as compared with 3.6 and 1.4 for β-2-thienylalanine and β-2-furylalanine, respectively. Concentrations of phenylalanine up to 100 mg per cent failed to reverse the inhibitory effect of α-amino-phenylmethane sulfonic acid.

When sorbitan monooleate medium was used as the basic substrate, complete bacteriostasis was produced by β-3-thienylalanine at 1 mg per cent, there being

a sharp rise in activity of the compound above the 0.25 mg per cent level (figure 1). Correspondingly lower concentrations of phenylalanine were required to neutralize the toxic effect of the analogue, but the antibacterial index at the various levels approximated that obtained with pellicle growth on the Proskauer-Beck medium. Table 3 gives data on the effect of different concentrations of β-3-thienylalanine and phenylalanine on the growth of the H37Rv tubercle bacillus.

TABLE 3

Growth of tubercle bacilli in sorbitan monooleate medium containing β-3-thienylalanine and phenylalanine

CONCENTRATION OF β-3-THIENYLALANINE	CONCENTRATION OF PHENYLALANINE	OPTICAL DENSITY $\times$ 10
mg %	*mg %*	
0	0	1.35
0.1	0	1.35
0.25	0	0.40
0.5	0	0.15
1.0	0	0
2.5	0	0
5.0	0	0
0	0.1	1.30
0	0.5	1.40
0	1.0	1.35
0	2.5	1.35
0	5.0	1.35
1.0	0.1	0
1.0	0.25	0.45
1.0	0.5	0.90
1.0	1.0	1.35
2.5	0.1	0
2.5	0.5	0.15
2.5	1.0	1.00
2.5	2.5	1.35
5.0	0.5	0
5.0	1.0	0.35
5.0	2.5	1.15
5.0	5.0	1.35

In addition to phenylalanine, five other amino acids also were able to neutralize the inhibition produced by β-3-thienylalanine. Of these, tyrosine was the most effective, with higher concentrations of leucine, isoleucine, norleucine, and tryptophan being required to produce a comparable effect. Fifteen mg per cent of tyrosine neutralized the effect of 5 mg per cent of β-3-thienylalanine, in comparison with 5 mg per cent of phenylalanine and 50 mg per cent of the other effective amino acids.

DISCUSSION

Phenylalanine is among the amino acids synthesized by the tubercle bacillus (Tamura, 1913). The finding that three of the phenylalanine analogues tested

served as inhibitors in the metabolism of this amino acid in the tubercle bacillus is further evidence that even in organisms that have the inherent ability of elaborating their own amino acids and growth factors, a nutritional deficiency may be produced by a blocking of either the synthesis or the utilization of an essential metabolite.

β-3-Thienylalanine was found to be the most effective inhibitor tested and may be classified as a true "antiphenylalanine" since phenylalanine was more active in neutralizing the inhibitory action of the analogue than any of the other amino acids. The finding that tyrosine, leucine, isoleucine, norleucine, and tryptophan are also effective in this respect is interesting in view of similar findings by Dittmer et al. (1946) and Clark and Dittmer (1948) in regard to their ability to neutralize inhibition in Saccharomyces cerevisiae and Escherichia coli produced by β-2-thienylalanine and β-2-furylalanine. The mode of action of these amino acids in the phenylalanine metabolism of the tubercle bacillus is unknown, but they may possibly serve as precursors in phenylalanine synthesis just as tryptophan has been suggested as a precursor of phenylalanine for E. coli by Beerstecher and Shive (1946).

The difference in activity between β-2-thienylalanine and β-3-thienylalanine emphasizes again the importance of molecular configuration in the determination of the antibacterial activity of a compound. The greater activity of β-3-thienylalanine would seem to justify additional studies with this antagonist.

SUMMARY

Five analogues of phenylalanine have been tested in vitro on the H37Rv strain of tubercle bacillus and the degree of inhibition determined. β-3-Thienylalanine, β-2-thienylalanine, β-2-furylalanine, and α-amino-phenylmethane sulfonic acid inhibited growth of the organism, but the activity of only the first three compounds was neutralized by phenylalanine.

β-3-Thienylalanine was the most toxic compound tested, 5 mg per cent completely preventing growth in the Proskauer-Beck medium, and 1 mg per cent in the sorbitan monooleate medium.

In addition to phenylalanine, tyrosine, leucine, isoleucine, norleucine, and tryptophan counteracted the toxicity of β-3-thienylalanine although higher concentrations of these amino acids were required.

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THE IN VIVO ACTIVITY OF NEOMYCIN[1,2]

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Among the most important properties of a new antibiotic are its toxicity to animals and its *in vivo* activity. When this antibiotic is produced by a member of a group of organisms that has already yielded several important or promising agents, a comparison of their respective *in vivo* activities and chemotherapeutic potentialities is desirable. Since neomycin (Waksman and Lechevalier, 1949) is produced by a member of the genus *Streptomyces*, which has yielded such interesting and important compounds as streptothricin, streptomycin, chloromycetin, and aureomycin, it is logical that the comparison should be based upon these agents, especially the first two, which were isolated in this laboratory.

Neomycin differs from streptothricin and from streptomycin in its antibiotic spectrum. Although streptothricin has potential *in vivo* activity (Metzger *et al.*, 1942), its latent toxicity prevents its use as a chemotherapeutic agent. Streptomycin, on the other hand, is not very toxic (Jones *et al.*, 1944) and is highly effective against certain gram-positive and gram-negative bacteria. To protect mice against infection with *Salmonella schottmülleri* and egg embryos against *Salmonella pullorum*, 50 μg per mouse or embryo were required.

Neomycin is slightly more effective against such bacteria than is streptomycin. Mice weighing 15 to 20 g require doses as low as 25 units and even 12.5 units to protect them against *Staphylococcus aureus;* concentrations of 100 units or less were effective against *S. schottmülleri*. Both streptomycin-sensitive and streptomycin-resistant strains were found to be affected alike by neomycin both *in vivo* and *in vitro* (Waksman and Lechevalier, 1949).

The toxicity of neomycin to mice, when given in a single subcutaneous injection, was found to range from above 2,000 to above 5,000 units per mouse, depending on the nature of the particular lot of material and the degree of purity. Crude preparations of neomycin instilled into the eye sac of the rabbit in concentrations of 1,500 units per ml for 30 minutes produced only a mild transient irritation. No delayed toxic signs were noticed during the 7-day observation period.

EXPERIMENTAL RESULTS

Effect against S. aureus. The following results of detailed investigations of the *in vivo* activity of neomycin, as compared to streptomycin, tend to substantiate previous conclusions and enlarge the number of organisms tested.

The comparative effects of the two antibiotics upon *S. aureus* are illustrated in table 1. The sterile preparations were given as single subcutaneous doses following the intraperitoneal administration of a 6-hour-old culture. It required 12.5 μg of streptomycin to bring about 95 per cent protection of the mice. In the case of neomycin, however, 3 units protected 100 per cent of the mice, whereas 0.75 and 1.5 units per mouse brought about 60 and 70 per cent protection, respectively. Although the effects of the two antibiotics against *S. aureus* in mice

TABLE 1

Efficacy of neomycin against S. aureus

Infection—*S. aureus* sm in 4 per cent mucin injected intraperitoneally.

Treatment—Aqueous solution of antibiotics injected subcutaneously immediately after infection.

NUMBER OF MICE	DILU-TION OF CUL-TURE	TREATMENT	UNITS PER MOUSE*	SURVIVAL OF MICE IN DAYS						PER CENT SURVIVAL
				1	2	4	6	8	10	
25	10^{-3}	Controls	—	0	—	—	—	—	—	0
15	10^{-4}	Controls	—	1	1	1	1	1	1	7
15	10^{-5}	Controls	—	3	3	3	3	3	3	20
15	10^{-6}	Controls	—	11	9	9	9	9	9	60
10	10^{-3}	Streptomycin	1.5	0	—	—	—	—	—	0
20	10^{-3}	Streptomycin	3.1	3	3	3	3	3	3	15
20	10^{-3}	Streptomycin	6.2	4	3	2	2	2	2	10
20	10^{-3}	Streptomycin	12.5	20	20	20	19	19	19	95
15	10^{-3}	Streptomycin	25.0	15	15	15	15	15	15	100
15	10^{-3}	Streptomycin	50.0	15	15	15	15	15	15	100
10	10^{-3}	Neomycin	0.17	0	—	—	—	—	—	0
10	10^{-3}	Neomycin	0.35	0	—	—	—	—	—	0
10	10^{-3}	Neomycin	0.75	8	6	6	6	6	6	60
10	10^{-3}	Neomycin	1.5	7	7	7	7	7	7	70
10	10^{-3}	Neomycin	3.1	10	10	10	10	10	10	100
10	10^{-3}	Neomycin	6.25	10	10	10	10	10	10	100
10	10^{-3}	Neomycin	12.5	10	10	10	10	10	10	100
10	10^{-3}	Neomycin	25.0	10	10	10	10	10	10	100
10	10^{-3}	Neomycin	50.0	10	10	10	10	10	10	100

* One unit = 1 μg in the case of streptomycin.

were markedly similar, neomycin is effective in one-quarter or less the dose required for streptomycin.

When a streptomycin-resistant strain of *S. aureus* was used for inoculation of the experimental animals, the results were even more striking. It required 1,000 μg of dihydrostreptomycin to protect 90 per cent of the mice, whereas neomycin brought about 100 per cent protection in concentrations as low as 12.5 units (table 2).

When administered orally, in a single dose, neomycin in concentrations of 200 to 400 units per mouse gave 60 to 90 per cent protection against *S. aureus*; similar concentrations of streptomycin, however, gave only 30 to 80 per cent protection (table 3). Subcutaneously, however, a much smaller concentration of neomycin,

TABLE 2

Comparative effects of neomycin and dihydrostreptomycin upon mice infected with dihydro-streptomycin-resistant S. aureus

Infection—intraperitoneally in 4 per cent mucin.

Subcutaneous treatment—single dose administration of antibiotic.

NUMBER OF MICE	DILUTION OF CULTURE	TREATMENT	UNITS PER MOUSE	SURVIVAL OF MICE IN DAYS				PER CENT SURVIVAL
				1	2	4	10	
10	10^{-2}	Controls	—	0	—	—	—	0
10	10^{-3}	Controls	—	0	—	—	—	0
10	10^{-4}	Controls	—	10	8	8	7	70
10	10^{-2}	Dihydrostreptomycin	50	0	—	—	—	0
10	10^{-2}	Dihydrostreptomycin	1,000	10	9	9	9	90
10	10^{-2}	Dihydrostreptomycin	5,000	10	10	10	9	90
4	10^{-2}	Neomycin	12.5	4	4	4	4	100
4	10^{-2}	Neomycin	25	4	4	4	4	100
4	10^{-2}	Neomycin	50	4	4	4	4	100

TABLE 3

Oral and subcutaneous efficacy of neomycin against S. aureus

Infection—*S. aureus* sm in 4 per cent mucin injected intraperitoneally.

Treatment—Aqueous solution of antibiotics administered orally or subcutaneously (SC), as indicated, immediately after infection.

NUMBER OF MICE	DILUTION OF CULTURE	TREATMENT	UNITS PER MOUSE	SURVIVAL OF MICE IN DAYS						PER CENT SURVIVAL
				1	2	4	6	8	10	
10	10^{-3}	Controls	—	0	—	—	—	—	—	0
10	10^{-4}	Controls	—	1	1	1	1	1	1	10
10	10^{-5}	Controls	—	6	6	6	6	6	6	60
10	10^{-6}	Controls	—	7	7	7	7	7	7	70
10	10^{-3}	Streptomycin (oral)	50	0	—	—	—	—	—	0
10	10^{-3}	Streptomycin (oral)	100	0	—	—	—	—	—	0
10	10^{-3}	Streptomycin (oral)	200	3	3	3	3	3	3	30
10	10^{-3}	Streptomycin (oral)	400	8	8	8	8	8	8	80
10	10^{-3}	Streptomycin (oral)	800	10	10	10	10	10	10	100
10	10^{-3}	Streptomycin (SC)	1.5	0	—	—	—	—	—	0
10	10^{-3}	Streptomycin (SC)	3.1	3	3	3	3	3	3	30
10	10^{-3}	Streptomycin (SC)	6.25	3	3	3	3	3	3	30
10	10^{-3}	Streptomycin (SC)	12.5	8	7	7	7	7	7	70
10	10^{-3}	Streptomycin (SC)	25.0	10	10	10	10	10	10	100
10	10^{-3}	Neomycin (oral)	50	0	—	—	—	—	—	0
10	10^{-3}	Neomycin (oral)	100	0	—	—	—	—	—	0
10	10^{-3}	Neomycin (oral)	200	6	6	6	6	6	6	60
10	10^{-3}	Neomycin (oral)	400	9	9	9	9	9	9	90
10	10^{-3}	Neomycin (oral)	800	10	10	10	10	10	10	100
10	10^{-3}	Neomycin (SC)	3.1	1	1	1	1	1	1	10
10	10^{-3}	Neomycin (SC)	6.2	9	9	9	9	9	9	90
10	10^{-3}	Neomycin (SC)	12.5	10	10	10	10	10	10	100
10	10^{-3}	Neomycin (SC)	25.0	10	10	10	10	10	10	100
10	10^{-3}	Neomycin (SC)	50.0	10	10	10	10	10	10	100

namely, 6.2 units per mouse, gave 90 per cent protection in this experiment, whereas double the concentration of streptomycin yielded only 70 per cent protection.

Effect against S. schottmülleri. A highly infective strain of *S. schottmülleri* was used in determining the comparative effects of neomycin and streptomycin. Here again, much lower concentrations of neomycin were required to give protection. A dose of 50 units of neomycin gave 90 per cent protection, wheras 200 units of streptomycin caused only 86 per cent protection, as shown in table 4.

When a streptomycin-resistant strain was used, the results were even more striking, as brought out in table 5. As high a dose as 5,000 µg of streptomycin

TABLE 4

Efficacy of neomycin against S. schottmülleri

Infection—*S. schottmülleri* in 4 per cent mucin injected intraperitoneally.

Treatment—Aqueous solution of antibiotics injected subcutaneously immediately after infection.

NUMBER OF MICE	DILUTION OF CULTURE	TREATMENT	UNITS PER MOUSE	SURVIVAL OF MICE IN DAYS						PER CENT SURVIVAL
				1	2	4	6	8	10	
15.	10^{-4}	Controls	—	4	1	0	—	—	—	0
10	10^{-5}	Controls	—	5	0	—	—	—	—	0
10	10^{-6}	Controls	—	5	2	2	1	1	1	10
10	10^{-7}	Controls	—	7	2	—	—	—	—	0
10	10^{-4}	Streptomycin	25	0	—	—	—	—	—	0
10	10^{-4}	Streptomycin	50	1	0	—	—	—	—	0
10	10^{-4}	Streptomycin	75	4	0	—	—	—	—	0
15	10^{-4}	Streptomycin	100	15	8	7	5	4	4	27
15	10^{-4}	Streptomycin	200	15	14	14	13	13	13	86
15	10^{-4}	Streptomycin	400	15	15	15	15	15	15	100
10	10^{-4}	Neomycin	25	5	3	1	—	—	—	0
10	10^{-4}	Neomycin	50	10	10	9	9	9	9	90
10	10^{-4}	Neomycin	75	10	10	10	10	10	10	100 .
10	10^{-4}	Neomycin	100	10	10	10	10	10	10	100

per mouse had only a slight effect in protecting the animals against the streptomycin-resistant culture, since only 40 per cent of the animals survived. In the case of neomycin, however, 50 units were sufficient to give 100 per cent protection, and 25 units gave 80 per cent protection.

The results of a typical experiment on the effect of neomycin upon streptomycin-sensitive and streptomycin-resistant strains of *S. schottmülleri* using egg embryos as the experimental animals are also reported (table 6).

Ten-day-old egg embryos were inoculated with 24-hour cultures of the two organisms grown in brain-heart infusion broth. The number of viable cells in the washed saline suspension was 10^{12} per ml. For inoculation purposes, 0.2 ml of a 10^{-2} or 10^{-4} dilution in 0.85 per cent saline were used. Inoculation was made into the chorioallantoic cavity, aseptic precautions being taken, through a hole drilled

TABLE 5

Efficacy of neomycin against S. schottmülleri (streptomycin-resistant)

Infection—*S. schottmülleri* (streptomycin-resistant) in 4 per cent mucin injected intra-peritoneally.

Treatment—Aqueous solution of antibiotics administered subcutaneously immediately after infection.

NUMBER OF MICE	DILUTION OF CULTURE	TREATMENT	UNITS PER MOUSE	SURVIVAL OF MICE IN DAYS						PER CENT SURVIVAL
				1	2	4	6	8	10	
15	10^{-3}	Controls	—	5	0	—	—	—	—	0
15	10^{-4}	Controls	—	12	10	8	7	6	5	33
15	10^{-5}	Controls	—	15	15	13	13	13	11	73
15	10^{-6}	Controls	—	15	15	15	14	14	14	93
15	10^{-3}	Streptomycin	100	5	3	1	1	1	1	6
10	10^{-3}	Streptomycin	500	2	1	1	1	0	—	0
15	10^{-3}	Streptomycin	1,000	7	1	1	1	0	—	0
10	10^{-3}	Streptomycin	2,000	6	2	1	1	1	1	10
15	10^{-3}	Streptomycin	5,000	15	9	8	8	7	6	40
10	10^{-3}	Neomycin	12.5	8	6	6	6	5	4	40
10	10^{-3}	Neomycin	25	10	10	10	10	9	8	80
10	10^{-3}	Neomycin	50	10	10	10	10	10	10	100
15	10^{-3}	Neomycin	100	15	15	15	15	15	15	100
15	10^{-3}	Neomycin	200	15	15	15	15	15	15	100

TABLE 6

Effect of neomycin upon streptomycin-sensitive and -resistant strains of S. schottmülleri injected into chick embryos

NUMBER OF EGGS	DILUTION OF CULTURE	TREATMENT	UNITS PER MOUSE	SURVIVAL OF EMBRYOS IN DAYS				PER CENT SURVIVAL
				1	2	4	8	
S. schottmülleri—streptomycin-sensitive								
5	10^{-2}	Controls	—	1	0	—	—	0
5	10^{-4}	Controls	—	0	0	—	—	0
10	10^{-2}	Neomycin	100	5	5	5	4	40
10	10^{-2}	Neomycin	200	6	6	5	5	50
10	10^{-4}	Neomycin	50	9	7	7	6	60
10	10^{-4}	Neomycin	100	9	9	9	8	80
10	10^{-4}	Neomycin	200	9	9	9	8	80
S. schottmülleri—streptomycin-resistant								
5	10^{-2}	Controls	—	2	2	1	1	20
5	10^{-4}	Controls	—	3	3	2	1	20
10	10^{-2}	Neomycin	100	6	6	4	4	40
10	10^{-2}	Neomycin	200	6	6	4	4	40
10	10^{-4}	Neomycin	50	7	7	7	7	70
10	10^{-4}	Neomycin	100	7	7	7	7	70
10	10^{-4}	Neomycin	200	8*	8*	8*	8*	80

' One nonspecific death.

in the shell over the air sac. Immediately following the bacterial injection, the eggs were treated with varying amounts of neomycin in 0.2 ml of distilled water, in a manner similar to that described above for the bacterial inoculation. Only crude preparations of neomycin containing 13 to 69 units per mg were available for these tests. The eggs were then returned to the incubator, and observations were made daily for 8 days or until the surviving embryos were ready to hatch. At the end of the incubation period the eggs were tested for sterility by the inoculation of 1 ml of the egg fluid into desoxycholate, lactose, sucrose medium. As little as 50 to 100 units of neomycin were required per embryo to give 70 per cent protection against the smaller number of bacteria. With larger numbers of bacterial cells used for infecting purposes (10^{-2} dilution), 100 units gave only 40 per cent protection. Those eggs that survived the 8-day incubation period were usually found to be sterile. There was little difference in the sensitivity of the two strains to neomycin.

Effect against Eberthella typhosa. Comparison of the effect of neomycin upon the infection of mice with *E. typhosa* gave even more striking results, as shown in table 7. Of neomycin, 50 units were sufficient to give 80 per cent protection, whereas even 400 μg of streptomycin, or eight times the dosage, gave only 50 per cent protection.

Effect against Salmonella pullorum. A more detailed study was made of the effect of neomycin upon the infectivity of egg embryos with *S. pullorum*. A culture of this organism obtained from the Food and Drug Administration laboratories was said to be more resistant to streptomycin than to streptothricin. The sensitivities of this culture to these two antibiotics and to neomycin were found to be >5.0, 1.0, and 0.5 units per ml, respectively. Neomycin is thus found to be most effective and streptomycin least.

The mode of inoculation and the subsequent treatment of the egg embryos were similar to those outlined previously. With a high concentration of bacterial cells (10^{-2} dilution) used for infection, 280 units of neomycin protected 50 per cent of the embryos; with a lower concentration of bacteria (10^{-4} dilution), however, 70 units were sufficient to protect the same percentage of the embryos (table 8).

In another experiment, crude neomycin (52 u per mg) was used to treat a series of egg embryos infected with 0.2 ml of a 10^{-4} dilution of a 24-hour-old culture of *S. pullorum*. The treatment took place immediately after infection, 3 hours later, and in varying concentrations after 3-, 6-, and 24-hour intervals. Only in the case of immediate treatment did 100 units of neomycin protect 50 per cent of the embryos; when used 3 hours after infection, only 30 per cent of the embryos were protected, with decreasing percentages upon prolongation of the interval between infection and treatment with the antibiotic. In another experiment it was found that 60 per cent of the embryos could be protected when the treatment (100 units) followed only 1 hour after infection.

The possible synergistic action of streptomycin and neomycin is brought out in table 9. Infected egg embryos were treated with different concentrations of streptomycin and neomycin, as well as with mixtures of the two antibiotics. Even

100 μg of streptomycin had no protective effect upon the infected embryos, whereas 25 units of neomycin protected 30 per cent of the embryos and 50 units exerted 70 per cent protection. The addition of 50 units of streptomycin to 25

TABLE 7

Effect of neomycin upon E. typhosa

Infection—*E. typhosa* injected intraperitoneally.

Treatment—Aqueous solution of antibiotics injected subcutaneously immediately after infection.

NUMBER OF MICE	DILUTION OF CULTURE	TREATMENT	UNITS PER MOUSE	SURVIVAL OF MICE IN DAYS						PER CENT SURVIVAL
				1	2	4	6	8	10	
10	10^{-2}	Controls	—	0	—	—	—	—	—	0
10	10^{-3}	Controls	—	0	—	—	—	—	—	0
10	10^{-4}	Controls	—	5	1	—	—	—	—	0
10	10^{-5}	Controls	—	6	6	3	0	—	—	0
10	10^{-2}	Streptomycin	25	1	0	—	—	—	—	0
10	10^{-2}	Streptomycin	50	9	7	4	3	1	1	10
10	10^{-2}	Streptomycin	100	10	10	7	1	—	—	0
10	10^{-2}	Streptomycin	200	10	10	6	3	1	1	10
10	10^{-2}	Streptomycin	400	10	10	9	7	5	5	50
10	10^{-2}	Neomycin	6.25	1	1	1	1	1	1	10
10	10^{-2}	Neomycin	12.5	9	8	7	5	4	2	20
10	10^{-2}	Neomycin	25	10	10	9	5	2	1	10
10	10^{-2}	Neomycin	50	10	10	10	10	9	8	80
10	10^{-2}	Neomycin	100	10	10	10	10	10	9	90

TABLE 8

Effect of neomycin upon S. pullorum injected into chick embryos

NUMBER OF EGGS	DILUTION OF CULTURE	TREATMENT	UNITS PER EMBRYO	SURVIVAL OF EMBRYOS IN DAYS				PER CENT SURVIVAL
				1	2	4	8	
10	10^{-2}	Controls	—	6	1	0	—	0
10	10^{-4}	Controls	—	8	2	0	—	0
10	10^{-6}	Controls	—	9	7	0	—	0
10	10^{-2}	Neomycin	280	8	7	6	5	50
10	10^{-2}	Neomycin	700	9	7	7	6	60
10	10^{-4}	Neomycin	70	9	8	6	5	50
10	10^{-4}	Neomycin	140	9	9	8	6	60
10	10^{-4}	Neomycin	280	9	9	9	8	80

The eggs receiving 140 or more units of neomycin gave negative results when tested for the presence of living *S. pullorum*. Eggs receiving 70 units of neomycin showed 20 per cent positive cultures.

units of neomycin raised the protective effect of the latter to 60 per cent, thus pointing to the potential synergistic action of the two antibiotics.

The bactericidal effect of neomycin upon *S. pullorum*, as measured by the degree of infection of egg embryos, is demonstrated by the results of the following

experiment. The bacterial culture was mixed with neomycin and allowed to incubate for varying periods, after which aliquot portions were injected into

TABLE 9

Use of a mixture of neomycin and streptomycin in protecting chick embryos (11 days) infected with S. pullorum

Infection—A single dose of a 24-hour-old culture of *S. pullorum* injected into the allantoic cavity; 0.2 ml of a 10^{-2} dilution in M/10 phosphate buffer was used.

Treatment—A single dose into the allantoic cavity given immediately after bacterial inoculation; streptomycin (S), in distilled water, equivalent to 50 or 100 μg; neomycin (N), in distilled water, equivalent to 25, 50, or 100 units; also a mixture equivalent to 25 units N + 50 units S and 50 units N + 50 units S. Dilutions made in distilled water; all inoculations in 0.2-ml doses.

Ten eggs were used in each test, unless otherwise noted; all surviving eggs gave upon subculture negative tests for *S. pullorum*.

DILUTION OF CULTURE	TREATMENT	UNITS PER EMBRYO	SURVIVAL OF EMBRYOS IN DAYS					PER CENT SURVIVAL
			1	2	4	6	8	
10^{-2}	0	—	10	1	0	—	—	0
10^{-4}	0	—	9	8	2	0	—	0
10^{-2}	S	50	10	7	4	1	0	0
10^{-2}	S	100	10	6	3	1	0	0
10^{-2}	N	25	10	7	7	6	3	30
10^{-2}	N	50	10	8	7	7	7	70
10^{-2*}	N	100	7	7	6	5	5	70
10^{-2}	N + S	25 + 50	10	9	8	8	6	60
10^{-2}	N + S	50 + 50	10	9	7	7	7	70

* Seven eggs only.

TABLE 10

Bactericidal effect of neomycin upon S. pullorum as determined by infection of egg embryos

A mixture of 0.2 ml of a 10^{-2} dilution of 24-hour-old culture in 0.85 per cent saline with 0.2 ml of 100 units neomycin in distilled water was allowed to stand for varying periods at 37 C, then injected into the chorioallantoic cavity of ten 10-day-old chick embryos.

NUMBER OF EMBRYOS	INCUBATION OF MIXTURE OF BACTERIA AND NEOMYCIN FOR HOURS	SURVIVAL OF EMBRYOS IN DAYS				PER CENT SURVIVAL
		1	2	4	8	
10	Controls	5	3	0	—	0
10	0	7	6	5	3	30
10	1	7	7	4	4	40
10	2.5	9	9	6	6	60
3	3.5	3	3	3	3	100

egg embryos. The results (table 10) show that neomycin has a marked bactericidal effect when allowed to remain in contact with the bacteria for even a very short time.

DISCUSSION

All the investigations reported here were conducted with rather crude preparations of neomycin. These varied in potency from 30 to 100 units per milligram of preparation. Judged by analogy to other antibiotics produced by members of the genus *Streptomyces*, notably streptothricin and streptomycin, these preparations can be considered as not more than 10 to 20 per cent pure. What effect the removal of excess impurities will have upon the activity of this antibiotic is difficult to foretell. It certainly should not reduce its potency but may reduce its toxicity.

It is not known as yet how many fractions make up the neomycin complex and whether the different fractions will show different effects upon different bacteria, as was shown to be the case for penicillin and for streptomycin.

SUMMARY

Crude preparations of neomycin in concentrations of 2,000 to 5,000 units when injected subcutaneously into mice weighing 15 to 20 g were well tolerated by experimental animals. These concentrations are at least 20 to 50 times the protective concentration of this antibiotic.

Neomycin did not exert any serious toxic effects when instilled into the rabbit's eye, a toxicity test characteristic for streptothricin.

Neomycin was more effective than streptomycin in suppressing infection of mice with *Staphylococcus aureus*. It was as effective upon the streptomycin-resistant strains of this organism as upon the sensitive strains.

Neomycin was more effective than streptomycin by oral administration in protecting mice infected intraperitoneally with *S. aureus*.

Neomycin was more effective than streptomycin in suppressing infections caused in mice and in chick embryos by *Salmonella schottmülleri*. It was as effective upon streptomycin-resistant as upon streptomycin-sensitive strains.

Neomycin was far more effective than streptomycin in suppressing infection of chick embryos with *Salmonella pullorum*.

Neomycin was highly bactericidal upon *S. pullorum* as measured by injecting embryos with mixtures of 24-hour-old cultures of the organism and neomycin.

Neomycin proved to be highly effective, far more than streptomycin, upon *Eberthella typhosa* in mice.

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STUDIES ON THE METABOLISM OF PHOTOSYNTHETIC BACTERIA

IV. Photochemical Production of Molecular Hydrogen by Growing Cultures of Photosynthetic Bacteria

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The outstanding characteristic of nonsulfur purple bacteria (*Athiorhodaceae*) is the ability to reduce CO_2 photochemically in the presence of organic hydrogen donors (Gaffron, 1933, 1935). These organisms differ from the sulfur purple bacteria (*Thiorhodaceae*) in two major respects: (a) they are unable to use reduced sulfur compounds as hydrogen donors, and (b) they require preformed vitamins for growth (Hutner, 1944, 1946). Although some species of *Athiorhodaceae* tolerate the presence of oxygen or may even develop aerobically in the dark to some extent, growth is generally optimal under anaerobic conditions in the light (van Niel, 1944). In the absence of oxygen, growth occurs only if the cultures are illuminated. Under these circumstances, purple bacteria are remarkably efficient as compared with typical heterotrophic anaerobes; thus far no significant quantity of any metabolic product other than CO_2 has been found in the medium of luxuriant cultures grown on "physiological" substrates (Gaffron, 1933; Muller, 1933).

Muller (1933) reported that anaerobic growth of sulfur purple bacteria in media containing mineral salts and organic compounds more oxidized than carbohydrate was accompanied by a net production of CO_2. On the other hand, when using more reduced organic hydrogen donors, he found it necessary to supply CO_2 in order to obtain growth. These observations on the growth of sulfur purple bacteria appear to be descriptive for the growth of *Athiorhodaceae* also.

Except for CO_2, no other gaseous products of *photosynthetic* activity have been noted previously in purple bacteria. The anaerobic production of H_2 by resting cells of a sulfur purple bacterium grown in peptone media was described by Roelofsen (1935). In this case, H_2 evolution was observed only during a dark "autofermentation" and was considered an artifact associated with autolysis of the cells. It was later shown by Nakamura (1937, 1939) that resting cells of *Rhodobacillus palustris* (*Athiorhodaceae*) and *Chromatium minutissimum* (*Thiorhodaceae*) decompose formate, glucose, pyruvate, glycerol, and glycerophosphate anaerobically in the dark with the production of H_2 as an end product.

In the present paper, the formation of H_2 (and CO_2) in growing cultures of *Rhodospirillum rubrum* (*Athiorhodaceae*) is described. Hydrogen is produced by this organism during growth on certain oxidized substrates in synthetic or semi-

synthetic media under anaerobic conditions in the light. Experiments with resting cells derived from such cultures have demonstrated unequivocally that the H_2 evolution is a light-dependent reaction and that the yield of H_2 can exceed one mol per mol of substrate added (Gest and Kamen, 1949; Gest, Kamen, and Bregoff, 1949). The phenomenon is unusual and unexpected because of the simultaneous production of excess CO_2, which ordinarily is capable of acting as a hydrogen acceptor.

EXPERIMENTAL PROCEDURES AND RESULTS

Hydrogen production in synthetic media. The production of H_2 by growing cultures of *Rhodospirillum rubrum* (strain SI) was originally noted in the following medium: fumaric acid, 3 g (or DL-malic acid, 3.5 g); L-glutamic acid, 4 g; potassium citrate·H_2O, 0.8 g; biotin 25 μg; $MgSO_4$·$7H_2O$, 0.2 g; $CaCl_2$, 38 mg; KH_2PO_4, 16 mg; K_2HPO_4, 24 mg; distilled water, 1 liter.[2] The pH was adjusted to 7 with NaOH before autoclaving. Anaerobic cultures were prepared by completely filling small glass-stoppered reagent bottles with inoculated medium. No special precautions were taken to remove air dissolved in the medium before inoculation. The bottles were illuminated with incandescent lamps at a temperature of approximately 30 C and the cultures shaken once a day to disperse sedimented bacteria.

The formation of CO_2 and H_2 was manifested by the appearance of a considerable gas space at the top of the bottle after displacement of medium around the glass stopper; it was necessary to tape the stopper down, since the gas production was ordinarily sufficiently vigorous to expel it from the bottle. Identification of H_2 in the gas was readily accomplished by explosion with palladinized asbestos, prepared according to the directions of Treadwell and Hall (1928). The production of an alkali-insoluble gas that is combustible with air in the presence of palladinized asbestos can also be demonstrated using Smith fermentation tubes in the usual manner.

The possibility that a nonphotosynthetic hydrogen-producing contaminant was responsible for gas evolution was eliminated by the following evidence: (a) no organisms other than the characteristic spirillae could be observed microscopically in the cultures, (b) no growth or gas production occurred if the inoculated cultures were incubated in the dark, and (c) illuminated yeast extract agar shake cultures made from bottles showing gas production contained colonies of purple bacteria only.

General nutritional requirements. Biotin has recently been identified as the single *essential* organic growth factor for various strains of *R. rubrum* (Hutner, 1944, 1946). We have observed, however, that the addition of yeast extract (250 mg Difco yeast extract per liter) to the synthetic media invariably leads to much more rapid growth and consequently to an earlier appearance of H_2 and CO_2 in cultures of *R. rubrum* (SI). From the results of growth experiments with the foregoing and numerous other media with various organic hydrogen donors, it

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[2] This medium is a modification of a recipe suggested by Dr. S. H. Hutner in a private communication.

appears that yeast extract supplies as yet unknown compounds that are required in addition to biotin for optimal growth. The results obtained also suggest that the requirements for optimal growth may vary, depending on the nature of the carbon and nitrogen sources provided.

The standard procedure now used in this laboratory for obtaining active hydrogen-producing bacteria for resting cell experiments is as follows: A medium of the composition given below is sown with a generous inoculum from a stab culture in 1 to 2 per cent agar plus 0.3 per cent Difco yeast extract.[3] Composition of liquid medium: DL-malic acid, 3.5 g; L-glutamic acid, 4 g; sodium citrate· $5\frac{1}{2}H_2O$, 0.8 g; biotin, $5\mu g$; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 38 mg; KH_2PO_4, 120 mg; K_2HPO_4, 180 mg; Difco yeast extract, 250 mg; distilled water, 1 liter; pH adjusted to 7 with NaOH before autoclaving. With a stab inoculum as the starter, excellent growth is usually obtained in 3 to 4 days under anaerobic conditions in the light (at a temperature of about 30 C).[4] The use of a large liquid inoculum (>0.5 per cent) from peptone or yeast extract cultures is not recommended because of the inhibitory effect of these complex substances on H_2 formation.

It is of interest to note that the amount of phosphate required by *R. rubrum* (SI) for maximal growth is considerably less than that normally used in culture media. Separate experiments with several synthetic media have shown that the cell yields do not fall off appreciably until the phosphate level is reduced to less than 2 to 4 mg P per liter.

Substrates necessary for H_2 production. In an attempt to determine which organic components of the medium were required for H_2 evolution, growth experiments were conducted using media containing the individual substrates and the various possible combinations. Ammonium chloride (1 g per liter) was added in cases in which glutamic acid was omitted. Culture experiments of this kind indicated that H_2 was formed only if glutamic and fumaric acids were both present. The results were unaffected by the presence or absence of citrate, which was added simply as a complexing agent to prevent precipitation of insoluble compounds.[5]

R. rubrum (SI) grows equally well and produces H_2 in media of the composition already given but containing succinic or malic acids in place of fumarate. Preliminary experiments also indicate that aspartic acid may be substituted for glutamate as a nitrogen source; it is possible that other amino acids will be found to be suitable.

Growth experiments using a "basal" glutamate-malate medium supplemented with different quantities of Difco yeast extract disclosed that H_2 formation is not observable when the concentration of yeast extract is 1 gram per liter or higher. Similarly, addition of NH_4Cl (0.5 to 2 g per liter) or Difco peptone (10 g per liter)

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[3] Freshly prepared medium of this composition is inoculated and incubated in the light to provide stock stab cultures. Good growth is obtained in 5 to 6 days.

[4] Moderately good growth is obtained under "semiaerobic" conditions also. Numerous other strains of *R. rubrum* will grow well and produce H_2 anaerobically in this medium.

[5] Citrate is not readily utilizable by *R. rubrum* (SI) as a carbon source for growth. It is not metabolized appreciably by resting cells under anaerobic conditions in the light.

to the synthetic medium abolishes hydrogen production. The inhibitory substances present in the yeast extract and peptone are as yet unknown. In connection with the effect of these materials, it is of interest that Gunsalus (1947) has reported the presence of unknown hydrogen acceptors in yeast extract which participate in the anaerobic fermentation of glycerol by *Streptococcus faecalis*. It is to be noted that, in all of these cases, the growth is abundant even though H_2 is not produced as a metabolic product. From these results it is evident that H_2 is found in growing cultures of *R. rubrum* only under certain specific conditions. Since NH_4Cl and yeast extract (or peptone) were routinely added to culture media for nonsulfur purple bacteria in the past, it is not surprising that the production of H_2 was not previously observed.

The tentative conclusion that both an amino acid and a dicarboxylic acid were required for H_2 evolution by *R. rubrum* (SI)[6] had been reached on the basis of the growth experiments described at the beginning of this section. However, in view of the ammonia inhibition it is apparent that the tests with ammonia as a nitrogen source nullified the conclusions drawn initially. Experiments with resting cells have shown that malic or fumaric acids alone can initiate the production of H_2 and that the phenomenon is completely inhibited by the addition of ammonia (Gest and Kamen, 1949; Gest, Kamen, and Bregoff, 1949). Succinic acid appears to be an exceptional substrate—in this particular case, the simultaneous presence of an amino acid seems to be necessary for H_2 production. In any event, this unusual combination of circumstances emphasizes the caution that must be exercised in attempting to deduce mechanisms from the results of growth experiments only.

It may be remarked in passing that a large number of substrates including fatty acids, sugars, purines, alcohols, and amino acids have been found to be ineffective in evoking photohydrogen production by resting cells (Gest, Kamen, and Bregoff, 1949).

Fixation of molecular nitrogen. An extremely interesting new aspect of the nitrogen metabolism of *R. rubrum* has been uncovered with the finding that this organism can incorporate molecular nitrogen into cell material when illuminated in a medium containing malate, biotin, and mineral salts (Kamen and Gest, 1949). This incorporation of N_2 is negligible if ammonia is added or if the organisms are incubated in the dark. These facts together with the observations that ammonia and molecular nitrogen inhibit photochemical H_2 formation by resting cells make it very probable that *R. rubrum* will be of great value for further investigation of the obscure relationship between hydrogenase activity and nitrogen fixation previously noted in other microorganisms (Wilson and Burris, 1947).

DISCUSSION

A more extensive investigation of the conditions required for observing H_2 formation in growing cultures is desirable but has been temporarily relinquished

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[6] This strain of *R. rubrum* is unable to utilize nitrate as a nitrogen source and differs from the strain studied by Hutner (1944) in at least two respects—the latter can metabolize glucose and apparently cannot use ammonia.

in favor of resting cell experiments because of the difficulties already noted in interpreting the results of growth experiments. An investigation of the ubiquity of photochemical H_2 production in other species of nonsulfur purple bacteria and its possible occurrence in the sulfur purple bacteria will also be of decided interest from the viewpoint of comparative microbiology.

Previous work by Roelofsen (1935) with resting cell suspensions of a sulfur purple bacterium demonstrated a dark autofermentative evolution of H_2 that could not be augmented by the addition of various substrates. The relationship of Roelofsen's observations to those reported here is not clear. It is probable that H_2 can be produced by purple bacteria by several mechanisms, some of which are entirely independent of light. This opinion is further supported by the report that *Rhodobacillus palustris* and *Chromatium minutissimum* produce H_2 from formate, glucose, pyruvate, glycerol, and glycerophosphate in the dark (Nakamura, 1937, 1939). We have also observed dark production of H_2 from formate by *R. rubrum* (SI); if the organisms are grown in the presence of formate, they will decompose this compound under an atmosphere of N_2 without an appreciable adaptation period. The results of supplementary experiments with growing cultures and resting cell suspensions, however, make it very doubtful that formate (or pyruvate) is of significance as an intermediate in the photoproduction of H_2 (Gest, Kamen, and Bregoff, 1949).

Anaerobic production of H_2 from dicarboxylic acids by heterotrophic bacteria is well known (Barker, 1936, 1937; Tabachnick and Vaughn, 1948; Woods and Clifton, 1937). In these cases, other fermentation products such as volatile fatty acids are also formed. There is no evidence for products other than H_2, CO_2, and cells in the present instance. In fact, the equations frequently used for describing the metabolism, including growth, of purple bacteria are of the following type (Rabinowitch, 1945).

$$C_n H_{2n}(COOH)_2 + \frac{n-3}{2} CO_2 + \frac{n-1}{2} H_2O \rightarrow$$

$$\frac{3n+1}{2} (CH_2O) - \sim(18n + 4)\text{kcal.}$$
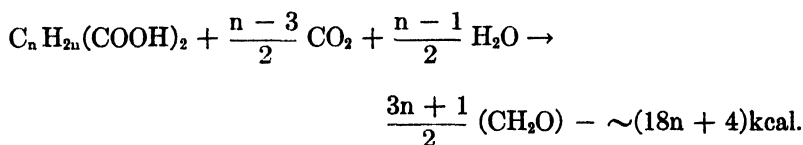
Although this type of formulation may predict the order of magnitude of CO_2 evolution or consumption to be expected under certain conditions, it is obviously of questionable significance when H_2 is also formed as a metabolic product.

The inhibitory effect of ammonia on H_2 formation implies (1) that molecular hydrogen may be a normal intermediate that can function as a hydrogen donor for reductive amination as well as for CO_2 reduction, or (2) that molecular hydrogen may be in equilibrium with a reduced compound HX that can act as a hydrogen donor in metabolism. A study of amino acid synthesis under various conditions in these bacteria is contemplated in the near future. A mechanism for the production of H_2 by purple bacteria and its relation to a similar phenomenon occurring in green algae (Gaffron and Rubin, 1942) will be discussed in a forthcoming publication (Gest, Kamen, and Bregoff, 1949).

SUMMARY

The photosynthetic bacterium *Rhodospirillum rubrum* has been found to produce molecular hydrogen in addition to CO_2 during anaerobic growth in a synthetic medium containing glutamate, fumarate, biotin, and mineral salts. Fumaric acid is replaceable by malate or succinate. Low concentrations of yeast extract effect a marked stimulation of growth and gas production, presumably by furnishing unknown substances required for optimal development. The photoproduction of hydrogen is inhibited by ammonia and high concentrations of yeast extract and peptone. Observations on the inhibition of photohydrogen production have led also to the unexpected finding that *R. rubrum* is a nitrogen-fixing organism. The significance of these phenomena is discussed briefly, particularly in relation to the formation of hydrogen by other microorganisms.

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A METHOD FOR THE DETERMINATION OF THE RATE OF GROWTH OF TUBERCLE BACILLI BY THE USE OF SMALL INOCULA[1]

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Older observations on the nutritive requirements and metabolism of virulent tubercle bacilli do not have quantitative significance because of the lack of accurate methods for the estimation of the rate of growth. The recognition in recent years that these organisms grow just as well when submerged in liquid media as on the surface of liquid or solid media has eliminated some of the difficulties inherent in measuring the amount of growth of tubercle bacilli. This type of culture eliminates many technical problems and permits the use of uniform homogeneous inocula. Employing subsurface growth, two methods have been described for the quantitative estimation of the amount and rate of growth of virulent tubercle bacilli. The first involves the use of micro-Kjeldahl nitrogen determinations (Youmans, 1946; Sattler and Youmans, 1948). Though accurate, this method suffers from being somewhat laborious and requiring relatively large numbers of organisms. The second employs photometric turbidity readings on cultures grown in Dubos' medium (Wolinsky and Steenken, 1947; Smith, 1947). The disadvantage of the second method resides in the composition of the medium that must be employed. Dubos' medium contains polyoxyethylene sorbitan monooleate, a surface-active agent that promotes a more diffuse type of growth and permits turbidimetric measurements (Dubos and Davis, 1946). Unfortunately, this substance has been shown to have a retarding effect on the growth of virulent tubercle bacilli and, furthermore, may markedly affect the action of substances present in the medium on the multiplication of tubercle bacilli (Fisher, 1948; Wong, Hambly, and Anderson, 1947; Forrest, Hart, and Walker, 1947; Kirby and Dubos, 1947; Youmans and Youmans, 1948).

Because of the importance, for purposes of diagnosis, of the isolation of small numbers of tubercle bacilli from pathologic material, very small numbers of tubercle bacilli have been used as inocula to determine the suitability of various media for the initiation of growth. This method has also been used to investigate the various nutritive requirements of the organism. However, because of the manner in which it has been employed, only approximate indications of the rate of growth have been obtained. Use of very small inocula for such purposes has suffered also from the lack of appreciation on the part of many workers of the sampling error involved (Drea, 1942). In using this method to determine the suitability of various media for the growth of tubercle bacilli, the usual procedure has been to inoculate tubes of media with one or more quantities of suspensions of tubercle bacilli which varied from 10^{-1} to 10^{-10} mg. Following incubation at 37 C for some arbitrary period of time, the tubes have been examined to deter-

mine the presence of growth, and when it is present, rough estimate is frequently made of the amount. It is obvious that although such a method will tell which medium will support growth of the smallest number of tubercle bacilli, no accurate determination can be made of the actual speed of multiplication on any given medium or under any set of conditions. The lack of quantitative data from such experiments probably accounts in part for the lack of unanimity of opinion regarding the most suitable media for the isolation and growth of virulent tubercle bacilli. A simple, relatively accurate method for the determination of the rate of growth of virulent tubercle bacilli is one of the greatest needs in the field today.

The use of inocula containing different quantities of tubercle bacilli actually provides a solution to the problem, provided the rate of growth of the tubercle bacilli is the same regardless of the number of bacilli used to inoculate the tubes and provided a method can be devised for determining the time at which each inoculum grows up to a certain standard mass. The latter can be accomplished by recording the time at which growth with each inoculum first becomes visible. If the rates of growth of the inocula employed are the same, a linear relationship should be obtained when the time of first appearance of growth of each inoculum is plotted against the logarithm of the amount of inoculum employed. The slope of the straight line so obtained will represent the rate of growth of the tubercle bacilli and can be used for the calculation of the growth rate and, if desired, the generation time. Thus, relatively accurate comparisons could be made of the rate of growth of tubercle bacilli in different media and under different conditions. The present paper details the successful application of the foregoing principles to the estimation of the growth rate of virulent human type tubercle bacilli.

EXPERIMENTAL RESULTS

The practicability of the method for the estimation of the rate of growth of tubercle bacilli outlined in the introduction was tested in the following manner: Suspensions of 14- to 21-day-old cultures of the H37Rv strain of *Mycobacterium tuberculosis* var. *hominis* were prepared and standardized as previously described (Youmans and Karlson, 1947). From this suspension dilutions were made with 0.01 M phosphate buffer solution, pH 7.0, so that the inocula to be employed contained, respectively, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} mg tubercle bacilli wet weight. Dilutions can also be prepared directly from cultures grown in "tween" albumin medium. Ten tubes, each containing 5.0 ml of the modified Proskauer and Beck basal synthetic medium (Youmans, 1946), were inoculated with each of the 8 quantities of H37Rv, making a total of 80 tubes in all. In addition, two modifications of the basal medium, one containing 0.2 per cent crystalline bovine albumin and the other 10.0 per cent sterile beef serum, were inoculated in the same manner. All tubes were then incubated at 37 C and examined daily for the presence of the typical granular growth. The time of the first appearance of such growth for each inoculum in each medium was recorded and then plotted against the logarithm of the inoculum. The results are shown in figure 1. The actual quantities of tubercle bacilli may be used along the ordinate since they are proportional to the logarithms.

An examination of figure 1 reveals that a linear relationship does exist between the time of the first appearance of growth and the logarithms of the number of organisms used as inocula. This indicates that the rate of growth is the same regardless of the amount of inoculum employed. Furthermore, the slopes of the lines in figure 1 represent the actual rates of growth. These two conclusions can be demonstrated mathematically or can be illustrated as in figure 2. In figure 2 lines have been drawn, representing an arbitrary similar rate of growth for each
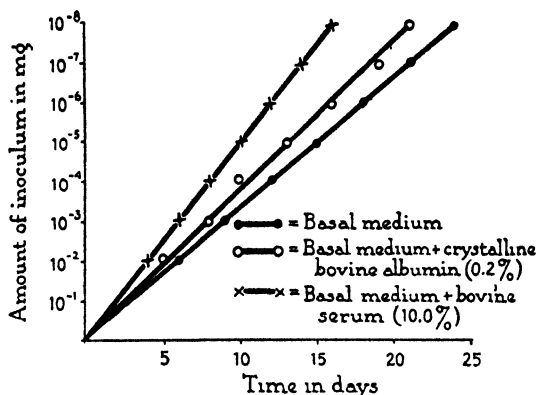
Figure 1. The rate of growth of *M. tuberculosis* (H37Rv) in three types of media.
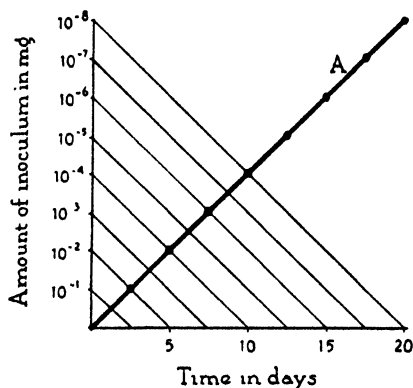
Figure 2. Diagrammatic representation of the estimation of the rate of growth of tubercle bacilli when using several small inocula.

amount of inoculum, until they intersect the abscissa. When the time in days, represented by the point of each intersection, is replotted against the logarithm of the respective inoculum, a straight line is obtained (line A), the slope of which is the same as the rate of growth of each inoculum. In such experiments the lack of a linear relationship between the time of appearance of growth and the logarithm of the inoculum would indicate either technical errors or actual differences in the rates of growth of the various inocula.

It should be borne clearly in mind that, when estimations are made of the time at which growth of each inoculum is first visible, we are in effect detecting in

each case approximately the same number of tubercle bacilli, i.e., the least num-
ber of bacilli that are visible. Therefore, in figure 1 an extension of the line should
intersect the ordinate at a point that will be equivalent to the least number of
tubercle bacilli that we can actually detect visually. When this is done with the
data in figure 1, we obtain a figure of 1.0 mg wet weight of tubercle bacilli per
5 ml of medium. Using suspensions of tubercle bacilli of known concentration,
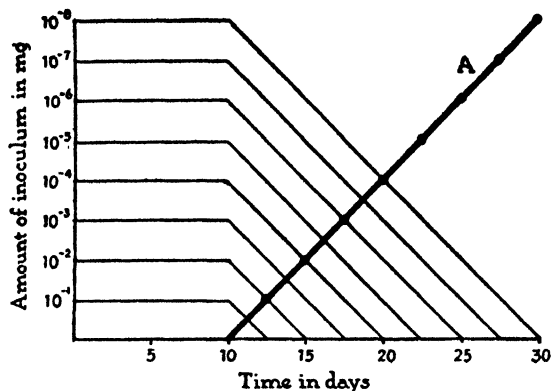
Figure 3. Diagrammatic representation of the effect of a lag phase of constant length on
the estimation of the rate of growth of tubercle bacilli when using several small inocula.
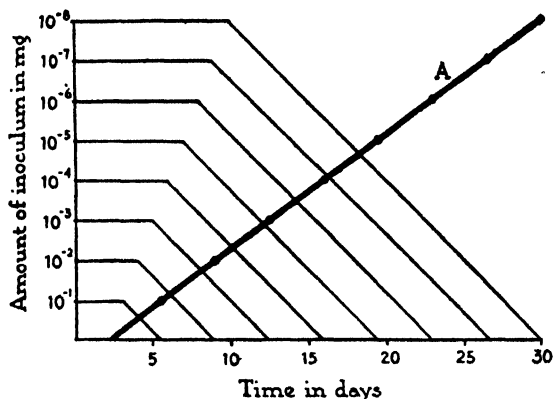
Figure 4. Diagrammatic representation of the effect of a lag phase the length of which
varies inversely with the amount of inoculum on the estimation of the rate of growth of
tubercle bacilli.

we have found that the least number of tubercle bacilli that are visible by the
method used is equivalent to approximately 0.5 mg per 5.0 ml of medium. The
difference between this value and the one obtained from the point of intersection
on the ordinate in figure 1 may be due to either experimental error or to the
presence of a lag phase of 1 or 2 days.

The influence of the lag phase on the accuracy of the method is illustrated in
figures 3 and 4. There are two possibilities to consider if a lag phase is present,
(1) that the length of the lag phase is constant regardless of the amount of inoc-

ulum, (2) that the length of the lag phase is inversely proportional to the logarithm of the amount of inoculum. The first possibility is illustrated diagrammatically in figure 3. In figure 3 an arbitrary lag phase of 10 days has been assumed for each inoculum. It is apparent that this does not affect the estimation of the rate of growth (line A). It affects only the point at which the line that represents the rate of growth intersects the abscissa. Furthermore, the time in days represented by this intersect gives the length of the lag phase. In figure 4 it has been assumed that the lag phase, if present, is inversely proportional to the logarithm of the amount of inoculum, and, in this case, an arbitrary increase of one day in the lag with each smaller inoculum has been assumed. When these data are treated as in figure 2, it can be seen that although a straight line is still obtained (line A) the slope of this line is less than the slope of the lines that represent the actual rate of growth after the lag phase has ended. The degree of divergence of the slope of the line from the true rate of growth will depend upon the degree to which the length of the lag phase is influenced by the number of organisms present. This, however, will not affect the accuracy of the estimations seriously unless this effect is marked. There is no information available on the influence of the number of tubercle bacilli on the length of the lag phase, but with other species of bacteria it is generally assumed that the lag phase, when present, is inversely proportional to the amount of inoculum (Winslow and Walker, 1939; Topley and Wilson, 1946; Porter, 1946).

In referring to the lag phase, care must be taken, however, to differentiate between increase in cell numbers and increase in cell mass. Hershey (1939) and Winslow and Walker (1939) have shown that when bacterial cells are inoculated into a favorable medium, the increase in cell mass is independent of the age of the culture and proceeds from the beginning at a constant rate, whereas the rate of cell division as measured by plate counts may indicate the presence of a lag phase. The method presented in this paper is essentially an estimation of the rate of growth by a measurement of bacterial mass. Therefore, the presence or absence of a lag in multiplication may possibly be of little significance.

Ingraham (1933) employed this same method in a study of the effect of gentian violet on the growth of 24 species of microorganisms other than mycobacteria. This author, however, thought that slopes of the straight lines so obtained were a function of the length of the lag phase. Herrington (1934) later, using the data reported by Ingraham, recognized that the slope of the line should be only a function of the rate of growth, but because of the fact that Ingraham stated that gentian violet affected growth only during the lag phase, he was forced to the conclusion that the length of the lag phase was affected by the amount of inoculum. Examination of the graphs given by Ingraham indicates that little or no lag phase was actually present. Unfortunately, Ingraham's conclusions that gentian violet affected *Escherichia coli* only during the lag phase were based on an enumeration of the number of cells by plate counts and direct counts, whereas measurement of the degree of effect of gentian violet on *E. coli* was made by the method herein described which, as has been pointed out previously, is a measure of the total mass of cells.

Kohn and Harris (1941), in their studies on the mode of action of sulfonamides, used a method for the estimation of the rate of growth of *E. coli* that was in principle similar to the one we have employed. These authors, using turbidimetric determinations, measured the time required for *E. coli* to multiply until a certain standard number of cells were present per ml of medium. They used the relation between these times to calculate the rate of growth. In the present work young actively growing cultures of tubercle bacilli were employed. It is well recognized (Winslow and Walker, 1939; Topley and Wilson, 1946; Porter, 1946) that, when actively growing cultures are transferred to a new medium, multiplication continues at the same rate.

From the data in figure 1 the slopes of the lines and therefore the growth rates were calculated by use of the following formula:

$$K = \frac{\log a - \log b}{t}$$

Where K = growth rate constant
a = largest inoculum employed (in mg)
b = smallest inoculum employed (in mg)
t = time in days or hours

The generation time (time for one cell division) was calculated in the following manner:

$$G = \frac{\log 2}{K}$$

Where G = generation time
K = growth rate constant

A total of six experiments similar to the one represented by figure 1 were done at different times. Table 1 gives the findings in detail of these six experiments, and figure 1 shows graphically the results obtained in one such experiment. The actual growth rate constants and generation times obtained in each experiment are given in table 2. The high degree of reproducibility of results using this method is apparent and the high degree of significance of the difference in growth rate between the basal medium and that medium containing crystalline bovine albumin indicates its usefulness. The tubercle bacilli actually grew on an average 13.0 per cent faster in the medium containing the bovine albumin. The marked stimulating effect of bovine plasma on the growth of tubercle bacilli is obvious, the average increase in rate over the basal medium being 27.6 per cent.

The technique as conducted in the experiments mentioned above is still laborious, involving as it does the preparation of 80 tubes of medium and the repeated handling of large numbers of tubes. This can be simplified considerably by employing fewer inocula. Actually, only two inocula would be necessary in order to establish the slope of the line and permit a calculation of the growth rate. For the sake of accuracy we prefer three; either 10^{-2}, 10^{-4}, and 10^{-6}, or 10^{-3}, 10^{-4}, and 10^{-6}. The number of tubes inoculated with each quantity of tubercle bacilli can be reduced, since sampling errors are not significant with

amounts of 10^{-6} mg or more. We now routinely use 5 tubes, since this number usually prevents technical errors or contamination from invalidating an experiment. This reduces the number of tubes for a growth rate estimation from 80 to only 15. The tubes, however, should be examined every day, or at the least

TABLE 1

Time in days of first appearance of growth of Mycobacterium tuberculosis (H37Rv) in three types of liquid media

AMOUNT OF IN-OCULUM IN MG	Basal medium						Basal medium with crystalline bovine albumin (0.2%)						Basal medium with beef serum (10.0%)					
	Experiment no.						Experiment no.						Experiment no.					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
10^{-1}		2	2	2	2	2		2	2	2	2	2		2	2	2	2	2
10^{-2}	6	5	5	5	4	5	5	4	5	5	4	4	4	4	4	4	4	4
10^{-3}	9	8	8	8	7	8	8	7	7	7	7	7	6	6	6	6	6	6
10^{-4}	12	11	11	10	10	10	10	9	9	9	9	9	8	8	8	8	8	8
10^{-5}	15	14	14	13	13	13	13	12	12	12	11	12	10	10	10	10	10	10
10^{-6}	18	16	17	16	16	16	16	14	14	14	14	14	12	12	12	12	12	12
10^{-7}	21	19	20	19	19	19	19	16	17	16	16	16	14	14	14	14	14	14
10^{-8}	24						21					18	16	16	16	16	16	16

TABLE 2

The rate of growth of the virulent human type tubercle bacillus (H37Rv) in three types of media

EXPERIMENT NO.	Basal medium		Basal medium plus crystalline bovine albumin 0.2%		Basal medium plus beef serum 10.0%	
	Growth rate constant (K)	Generation time in hours	Growth rate constant (K)	Generation time in hours	Growth rate constant (K)	Generation time in hours
1	0.333	21.7	0.375	19.3	0.5	14.4
2	0.375	19.3	0.428	16.9	0.5	14.4
3	0.375	19.3	0.428	16.9	0.5	14.4
4	0.352	20.5	0.428	16.9	0.5	14.4
5	0.375	19.3	0.428	16.9	0.5	14.4
6	0.375	19.3	0.428	16.9	0.5	14.4
Mean	0.364	19.90	0.419	17.3	0.5	14.4
Standard deviation		±1.0		±0.96		0.0
			$P = <0.01$			

every other day; if readings are made farther apart than this, gross errors are introduced.

The technique of examining the tubes for the presence of growth is important. Tubes of uninoculated medium should be used for comparative purposes. A constant source of light and a dark background are essential. We have found the

use of a Quebec colony counter ideal. This not only provides a constant light source and a dark background, but a small amount of magnification which facilitates the readings. With practice, the error is not more than plus or minus 1 day. Some objective method for the detection of a standard number of tubercle bacilli would be highly desirable, but, at present, because of the flocculent type of growth, this is not practical.

DISCUSSION

The method described herein for the estimation of the rate of growth of virulent tubercle bacilli is simple and relatively accurate. Although the method for the estimation of the presence of growth is subjective, the error is slight and the data can be plotted so that they have quantitative significance. The method is applicable to solid as well as liquid media and permits an accurate expression of the relative efficacy of different media for the growth of tubercle bacilli. This would tend to eliminate controversy regarding the relative merits of this or that medium, either for the growth of cultures of tubercle bacilli or for primary isolation. In the latter connection, it should be possible to dilute heavily positive sputum samples after concentration and estimate the rate at which bacilli from a human source will grow on any given medium at the time of isolation.

The usefulness of the method for the study of the nutritive requirements and metabolism of the tubercle bacillus is obvious. Subsequent papers will give in detail results of the application of the method to such studies.

Attention should be called to the discrepancy between the growth rates obtained with this method and those previously reported that were obtained by micro-Kjeldahl nitrogen determinations (Youmans, 1946; Sattler and Youmans, 1948). With the same basal medium, the shortest generation time obtained by the latter method was 38.0 hours, approximately twice as long as those obtained with the method reported herein. Although it is technically impossible to compare the two methods directly, it would appear that conditions are more favorable for the growth of minute inocula (10^{-1} to 10^{-8} mg) than for very large inocula (2 to 5 mg).

SUMMARY

Decimal dilutions of virulent human type tubercle bacilli were prepared which varied from 10^{-1} to 10^{-8} mg moist weight. Tubes of three types of liquid media were inoculated with each concentration of tubercle bacilli. These were incubated at 37 C and examined daily to determine the time at which visible subsurface growth of each inoculum first appeared. The determination of this point represented a measurement of approximately the same number of organisms in each set of tubes.

By plotting the logarithms of the inocula employed against the time at which visible growth of each inoculum first appeared, a linear relationship was found. From the slope of the straight line so obtained, the growth rate and the generation time could be calculated. This method can be used to determine with greater accuracy the effect of various substances or physical conditions on the rate of growth of tubercle bacilli.

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STREPTOMYCIN-DEPENDENT TUBERCLE BACILLI: A SIMPLE METHOD FOR ISOLATION

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This report describes a simple method for the isolation of streptomycin-dependent tubercle bacilli from virulent cultures that have not previously been exposed to streptomycin. The *in vivo* isolation of streptomycin-dependent *Mycobacterium tuberculosis* has been reported by Spendlove *et al.* (1948) and Lenert and Hobby (1949). The *in vitro* procedure described here is applicable to the quantitative determination of both the streptomycin-dependent and streptomycin-resistant variants.

EXPERIMENTAL PROCEDURE AND RESULTS

Tubercle bacilli from a stock culture of strain H37Rv were grown in a flask of liquid sorbitan monooleate albumin medium until a turbid growth was obtained. The flask was shaken well to obtain a uniform dispersion of cells, and the number of viable cells per ml was determined by the usual dilution and surface plating method using solid oleic acid albumin medium in the plates (Dubos and Middlebrook, 1947). To the remaining undiluted liquid culture, streptomycin was added to give a final concentration of 100 μg per ml of medium. Fifty plates, containing 100 μg of streptomycin per ml of solid oleic acid albumin medium, were each inoculated with 1 ml of the liquid culture containing approximately 280 million cells per ml.[1] The plates were incubated at 37 C and after 48 hours, when the excess moisture had evaporated, were sealed with wide rubber bands.

After 30 days' incubation, 62 colonies were found growing on the 50 plates. The individual colonies were circled with a wax pencil, and each colony was tested and found to be streptomycin-resistant. At that time, no streptomycin-dependent colonies were found. After 51 days' incubation, 22 additional colonies appeared. Of these 22 colonies, 15 were resistant to streptomycin, 5 were dependent on the presence of streptomycin for their growth, and the remaining 2 showed enhanced growth in the presence of streptomycin. The colonies of this second "crop" were marked and the plates were re-examined after 70 days' incubation. Twelve additional colonies were found, of which 4 were streptomycin-resistant, 7 streptomycin-dependent, and 1 showed increased growth in the presence of streptomycin.

Thus 50 plates, each inoculated with 280 million cells, were incubated for 70 days, and 96 colonies were found growing in the presence of 100 μg of streptomycin per ml of medium. There were 81 streptomycin-resistant colonies, 12 streptomycin-dependent colonies, and 3 colonies showing increased growth in the

[1] In two previous publications (Yegian and Vanderlinde, 1948, 1949) inocula size that read in billions per ml of medium should have read in millions per ml.

presence of streptomycin. The plates were incubated for longer than 70 days but no additional colonies appeared.

A similar experiment was performed using a strain of *Mycobacterium ranae*. As in the previous experiment, the culture was grown in liquid sorbitan mono-oleate albumin medium, but nutrient glycerol agar was used for solid medium. Fifty plates, containing 100 μg of streptomycin per ml of medium, were each inoculated with approximately 100 million cells from a 7-day-old undiluted culture and incubated at 37 C. The details of the procedure were similar to those described previously for the tubercle bacillus. After 7 days' incubation there was a total of 17 colonies, each of which was streptomycin-resistant. Following 14 days' incubation, there were 13 additional colonies, 8 of which were streptomycin-resistant and 5 streptomycin-dependent. After 21 days' incubation, 10 additional colonies appeared of which 2 were streptomycin-resistant and 8 streptomycin-dependent. Thus after 21 days' incubation of 50 plates, there were 40 colonies found growing in the presence of 100 μg of streptomycin per ml of medium. Of these, 27 were streptomycin-resistant and 13 streptomycin-dependent. These experiments were repeated twice and similar results were obtained each time.

At this time certain of the pertinent biological characteristics of this streptomycin-dependent variant of the tubercle bacillus will be described briefly. It was observed on the first two subcultures of the streptomycin-dependent strain of H37Rv that the growth rate in liquid medium was noticeably slow, but on the third subculture it was approximately the same as that of the streptomycin-sensitive and resistant strains. When the streptomycin-dependent strains were tested, using relatively small inocula, in liquid medium containing different concentrations of streptomycin, marked differences were noted in the rates of growth. Uniformly poor growth was noted in 1,000 μg of drug per ml of medium and good growth in 100, 50, and 10 μg per ml. A few strains showed poor growth in 1 μg of drug per ml; the others grew well in this concentration. In the absence of streptomycin, there was either no visible growth, very slow but progressive growth, or a very slight growth with no progression.

A dependent strain that showed no visible growth at 15 days in liquid medium in the absence of streptomycin was inoculated into a flask of liquid medium containing 100 μg of drug per ml, and after 15 days' incubation a good growth was obtained. The cells were separated from the medium by centrifugation and were then washed repeatedly with isotonic sodium chloride solution until the final suspension contained no detectable streptomycin. One-ml samples of this turbid suspension were placed on each of 54 plates containing solid medium without streptomycin. After 35 days' incubation there was an average of 8 colonies per plate growing in the absence of streptomycin. All of the colonies had typical cord formation (Middlebrook *et al.*, 1947). Streptomycin sensitivity tests in liquid sorbitan monooleate albumin medium were performed using 45 of these colonies selected at random. The results showed that all of the colonies tested were streptomycin-resistant. The virulence of 5 of these strains was tested and they were found to be pathogenic for guinea pigs.

DISCUSSION AND SUMMARY

The isolation of streptomycin-dependent microorganisms is not new, but a simple quantitative procedure for the isolation of streptomycin-dependent tubercle bacilli *in vitro* is of interest. In previous studies we were able to isolate streptomycin-dependent *Mycobacterium ranae* only from streptomycin-resistant strains under specific experimental conditions (Yegian and Budd, 1948). In later studies we were impressed by the delayed appearance of streptomycin-dependent colonies presumably originating from single cells (Yegian and Vanderlinde, 1948). On the basis of that observation, the present procedure was developed and it is now possible to isolate streptomycin-dependent *Mycobacterium tuberculosis* from strains that had not previously been exposed to the drug.

In contrast to streptomycin-dependent *M. ranae*, some strains of tubercle bacilli are strictly dependent on streptomycin, whereas others show only enhancement of growth in the presence of the drug. In addition, after several subcultures of streptomycin-dependent tubercle bacilli are made, the growth rate in liquid medium is essentially the same as that of the original parent and resistant strains. A third difference is the high incidence of streptomycin-resistant variants found in the streptomycin-dependent populations of *M. tuberculosis*. In contrast, the variants found in streptomycin-dependent populations of *M. ranae* were few in number and resembled the parent sensitive cells.

Detailed studies of the biological characteristics, including pathogenicity, of these streptomycin-dependent tubercle bacilli will be reported later.

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STRAIN DIFFERENCES IN ORAL LACTOBACILLI AND THE RELATION TO DENTAL CARIES

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Dental caries has been correlated by many investigators (Rosebury, 1944) with the presence of large numbers of lactobacilli in the saliva. These organisms produce a concentration of acid that is capable of dissolving the enamel of teeth (Fosdick and Starke, 1941; Bibby *et al.*, 1942). Sullivan (1939) reported that enamel becomes soft and pitted when placed in glucose broth inoculated with lactobacilli that produced a pH of 5 or less. No such changes were observed with lactobacilli that did not lower the pH below 5. However, Florestano (1942), studying a large number of individuals, found no correlation between the amount of acid produced in glucose broth and caries. Other factors than those directly related to the biochemical activities of the decay-producing bacterium may prevent a very close correlation. Another possible explanation is offered by Stephan and Hemmens (1947), who suggest that the concentration of the organisms, substrate, and buffer determines the final pH reached in a given length of time, and that this is probably quite different in the dental plaque from that in the usual culture medium.

Although most individuals with active caries have high lactobacillus counts in the saliva, some with no caries are frequently found that also have large numbers of the organism present in their saliva (Rosebury, 1944). This may mean that noncarious individuals harbor lactobacilli of a different type from those found in the saliva of carious individuals. Before a study of this possibility could be attempted, it was necessary to determine what characteristics of lactobacilli might be useful in classifying the organisms into groups or might be directly correlated with caries activity. Several attempts at typing or classifying the lactobacilli have been made with some degree of success. Hadley, Bunting, and Delves (1930) noted differences in the pH produced in 1 per cent glucose broth by various strains of lactobacilli obtained from scrapings from carious teeth. They grouped their organisms according to cell morphology and studied the fermentation of a large number of carbohydrates by these groups. Rosebury (1932) also observed differences in acid production between strains, as did Curran, Rogers, and Whittier (1933) and Harrison (1942). The latter investigators studied fermentation of several carbohydrates other than glucose. An extensive study of the biochemical interrelationships of the oral lactobacilli was made by Sullivan and co-workers (1939), who isolated 103 strains from extracted teeth. They divided the organisms into a weak-acid-producing and a strong-acid-producing group, which showed a rough correlation with salicin, raffinose, and mannitol fermentation.

The present investigation was made to determine what characteristics, if any,

of the oral lactobacilli might be found that were definitely associated with caries activity. This, it was hoped, might lead to an explanation of the presence of many lactobacilli in some noncarious mouths, and to a better understanding of the particular properties of the bacterium that make it more or less active as a caries producer. A study of 100 individuals in our own laboratory showed that, although lactobacillus counts were generally higher in the saliva of those individuals with caries than in the saliva of those with no caries, it was possible to isolate these bacteria from 27 who were diagnosed as being caries-free. This paper reports a further study of the biochemical properties of oral lactobacilli and the relation of these properties to caries activity.

Differences in acidogenic properties of oral lactobacilli. Saliva samples were cultured on tomato juice agar plates of pH 5 and the organisms picked to peptonized milk agar. Any gram-positive rod growing under these conditions was

TABLE 1

pH produced by lactobacilli grown in glucose broth

pH	NUMBER OF ORGANISMS
4.1 to 4.5	77
4.6 to 4.9	21
5.0 to 5.8	18
6.0 to 6.9	32

TABLE 2

Sugars fermented by the high and low acid-producing groups of lactobacilli

	SALICIN		RHAMNOSE		RAFFINOSE		MANNITOL	
	+	−	+	−	+	−	+	−
No. strains pH 5 or more..........	11	36	3	45	35	12	10	37
No. strains pH below 5..........	86	2	31	57	7	80	82	4

considered to be a lactobacillus. After the purity of the cultures was determined, they were maintained on peptonized milk agar and stored in the refrigerator. Before being tested for acidogenic properties they were transferred to peptonized milk agar, then to tryptose phosphate broth. Five-tenths ml of the broth culture were planted in flasks containing 100 ml of tryptose broth with 5 grams of glucose. The acid was titrated with N/10 NaOH and the pH was determined with a glass electrode after 96 hours. Longer periods of incubation produced no further lowering of the pH. The results of pH determinations on 148 strains are shown in table 1. The organisms may be divided into two groups: those that attained a pH of 4.9 or lower and those that did not attain a pH below 5 (the strong- and weak-acid-producing groups of Sullivan *et al.*, 1939). Titrations of the acid showed the low pH group to produce 40 ml or more of N/10 acid per 100 ml of medium, and the high pH group to produce less than 20 ml of acid.

Fermentation of sugars.   Preliminary work with 16 strains using glucose, maltose, lactose, sucrose, xylose, salicin, rhamnose, raffinose, and mannitol showed the last four to be the only carbohydrates with possibilities for classifying the organisms into groups.   All strains were then tested for ability to ferment these sugars.   The results are given in table 2.   Most of the strains could now be assigned to one of the three well-defined types given below:

    Type   I = pH below 5
                Salicin +
                Rhamnose +
                Mannitol +
                Raffinose − usually; a few strains +

    Type   II = pH below 5
                Salicin +
                Rhamnose −
                Mannitol +
                Raffinose − usually; a few strains +

    Type III = pH 5 or above
                Salicin −
                Rhamnose −
                Mannitol −
                Raffinose + usually; some strains −

Dehydrogenase activity.   Since acid production could be used as the basis for dividing the oral lactobacilli into two groups, which correlated with carbohydrate fermentations, and since the strong acid-producing group was divided into the rhamnose-positive and rhamnose-negative types, it seemed desirable to determine other metabolic characteristics that would possibly validate the divisions. Previous studies had shown 8 strains of oral lactobacilli to have dehydrogenase activity toward glucose, sucrose, maltose, lactic acid, pyruvic acid, formic acid, glycerol, and ethyl alcohol.   No activity was shown toward acetic acid and succinic acid.   There were differences in the degree of activity shown by different strains on some substrates.   Therefore glucose and maltose, and a hitherto untested carbohydrate (fructose), were used as substrates for the comparison of dehydrogenase activity of several newly isolated strains of lactobacilli.

The dehydrogenase activity was determined by the Thunberg technique, as described in a previous paper (Clapper, 1947).   Six type I strains, 4 type II strains, and 6 type III strains were used in preliminary work.   All of type III, the weak acid producers, showed very active dehydrogenases for glucose; all of type I of the strong acid producers also showed active dehydrogenases for glucose; and type II showed much less active dehydrogenases.   This was true with maltose and fructose, but not to such a striking degree as with glucose.

Since glucose gave the most definite differences in activities, other strains of the three types were tested.   Only those strains that gave typical acid production and fermentation reactions for the type were used.   The values in minutes required for complete decolorization of the methylene blue are given in table 3.

The values represent the average of at least 3 different determinations, all of which were done in duplicate. Although there is some overlapping in the times of decolorization for the three types, the organisms having the biochemical activities of type II are for the most part considerably less active than either type I or III lactobacilli. A difference in activity, which correlates well with the carbohydrate fermentations, is shown among the groups, as was indicated by the preliminary experiments.

Occurrence of the three types in the salivas of carious and noncarious individuals. Lactobacilli were isolated from the saliva of 69 different individuals, and from

TABLE 3

Glucose dehydrogenase activity of oral lactobacilli

pH BELOW 5 SALICIN +, MANNITOL +, RAFFINOSE −		pH ABOVE 5 SALICIN −, MANNITOL −, RAFFINOSE +
Type I rhamnose +	Type II rhamnose −	Type III
8*	20	3
10	33	4
10	42	5
10	49	5
10	50	7
15	53	10
17	60	10
20	60	15
20	63	15
20	75	17
22	80	20
22	105	35
23	150	
28	150	
40	195	
45	240	
47	>240	
49	>240	
Median = 20	Median = 63	Median = 10

* Time in minutes required to decolorize the methylene blue. Values represent the average of 3 determinations.

the cavities in, or plaques on, decayed teeth of 26 others. All of these people were examined by a dentist and were classified as follows: (1) noncarious—no cavities found at the time of the examination and no fillings for at least three months prior to the examination; and (2) carious—one or more open cavities at the time of examination.

At the outset of the experiment several organisms were isolated from each mouth. It was found that the majority of the lactobacilli from a given saliva sample had the same properties, and, although occasionally two different strains could be isolated from the same individual, we felt that little error would be in-

troduced by picking a single colony from a plate and assigning the strain found as the predominant one for the individual. This assumption was found to be statistically sound by picking 10 colonies from each of 10 different plates and subjecting the results to analysis. Three different groups of individuals were studied. The first varied in age from 6 to 40 years, with approximately one-third under 10, and almost all the remainder between the ages of 20 and 30 years. The second consisted of children living in Colorado Springs, who had been drinking water containing more than 1 ppm fluoride all of their lives. Their ages ranged from 6 to 13 years. The third were all carious, the organisms being obtained from the cavity or the plaque on the outside of the decayed tooth by transferring debris into glucose broth of pH 4 and then streaking a loopful of this on tomato juice agar. The ages of the individuals of this group were unknown. They were taken by the dentist from patients visiting his office for treatment, the only criterion for inclusion in the group being the presence of decay. The

TABLE 4

Occurrence of types of lactobacilli

	GROUP OBTAINED FROM SALIVA				GROUP OBTAINED FROM CAVITY OR PLAQUE ON DECAYED TOOTH
	Includes 50 picked at random and 19 from F area		Includes only those from area with F in the drinking water		
	69 Individuals		19 Individuals		26 Individuals
	33 Noncarious	36 Carious	12 Noncarious	7 Carious	26 Carious
% of					
Type I	9.1*	27.8	8.3	28.5	65.4
Type II	57.6	41.6	83.3	71.4	7.7
Type III	24.2	25.0	8.3	0.0	11.5
Irregular	9.1	5.6	0.0	0.0	15.4
Rhamnose +	9.0	30.5	8.3	28.5	69.2

* Values are expressed as the percentage of individuals showing the type indicated as the predominating organism.

number and percentage of types I, II, and III lactobacilli found in noncarious and carious individuals in each of these groups is shown in table 4.

After finding a significantly greater number of type I lactobacilli in carious mouths than in noncarious ones, single fermentations or combinations of fermentation with pH values were examined for possible correlation with the presence of caries. Rhamnose fermentation alone was found to have as high a positive correlation with caries as type I. This is shown by the values given in table 4.

DISCUSSION

The data given in table 1 show a wide variation in the acid-producing ability of the 148 lactobacilli studied. No direct correlation between this property and caries activity could be found. Table 2 shows that most of the low pH group ferment salicin and mannitol but not raffinose, the high pH group do not fer-

ment salicin and mannitol, and about 75 per cent ferment raffinose. This corresponds to the findings of Sullivan et al. (1939). By introducing the fermentation of rhamnose as an additional test, the high acid-producing group can be subdivided and the lactobacilli can be assigned to one of the three types previously indicated. Hadley et al. (1930) have reported data on a study of 18 carbohydrates, which shows that organisms capable of fermenting rhamnose can also ferment more of the other carbohydrates than can those unable to ferment rhamnose. Determination of glucose dehydrogenase activity proved that organisms of type I and type III were more active than those of type II. Such activity may result in a greater and faster accumulation of acid, or in the complete breakdown of the glucose with little or no accumulation of acid.

Examining each type of Lactobacillus for properties that might have a direct effect on the production of a carious lesion, we find that, although each type has some property or properties of this kind, type I has more of these than do types II and III. It shows (1) faster growth in liquid and solid media (greater than III), (2) ability to attain a low pH in glucose broth (greater than III), (3) ability to ferment more sugars (greater than III), (4) ability to ferment rhamnose (greater than II or III), and (5) active glucose dehydrogenase (greater than II).

The occurrence of these three types is shown in table 4. The data reveal that a larger percentage of individuals with cavities in their teeth have type I as the predominant kind of Lactobacillus. Applying the chi-square method with the Yates correction factor to the values found for the distribution of type I, the probability was approximately 0.02. Using the values given for type II, the probability was 0.25, and for Type III, 0.95. This means that the probability is very high that the greater number of type I lactobacilli found in carious mouths, as compared to noncarious mouths, is significantly related to caries incidence, that the greater number of type II found in noncarious mouths may possibly have some significance with regard to lack of caries, and that the distribution of type III is very likely to occur by chance and has no relation to caries.

The selected group from the area with F in the drinking water showed a similar predominance of type I in the carious mouths. The greater proportion of type II may be due to the influence of the F ion on the oral flora. When the organisms were obtained from cavities or plaques on decayed teeth, the percentage of type I lactobacilli found was greatly increased. Although we did not have organisms scraped from noncarious teeth for comparison, this is at least suggestive evidence that type I may be more responsible for caries production than are types II and III. This distribution may explain in part why some noncarious individuals show a high lactobacillus count in their saliva: the high count is made up of those types having less significance as caries producers. It also leads us to suspect that the type of Lactobacillus that becomes established as the predominant organism in a given mouth is determined by some factor in the oral environment peculiar to the carious or the noncarious individual. It has already been shown that caries activity is related to the number of organisms, so that caries may be produced from large numbers of less active organisms. A much higher correlation of type with caries activity might be realized, therefore, if

only those carious individuals with a moderate or low lactobacillus count were studied.

Attempts to correlate single carbohydrate fermentations, or combinations with pH, resulted in finding that rhamnose fermentation alone had a slightly higher correlation with dental caries than when the other properties of type I were included. Sixty-nine and two-tenths per cent of the 26 organisms isolated directly from plaques or lesions of 26 different individuals were the type of *Lactobacillus* that ferments rhamnose. The test for rhamnose fermentation is very easily performed and might be useful to include as an adjunctive test on those counts on saliva samples that show moderate to small numbers of organisms. A positive test on these may indicate that the individual is susceptible to caries, even though the lactobacillus count is not high. Further studies to confirm this relationship of type of *Lactobacillus* to caries by correlation with counts, and by studying specific age groups, are being planned.

ACKNOWLEDGMENTS

SUMMARY

The presence of dental caries has been found to be associated with a relatively high incidence of a specific type of *Lactobacillus* in the saliva and in dental plaques. This type of *Lactobacillus* produces a pH of less than 5 on glucose broth, has an active dehydrogenase for glucose, ferments salicin, mannitol, and rhamnose but not raffinose, and grows rapidly in broth.

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NOTES

AMINO ACIDS IN HEALTHY CHLORELLA CELLS

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The amino acids present in a hydrolyzate of healthy *Chlorella* cells were determined qualitatively by the technique of partition chromatography on paper described by Consden, Gordon, and Martin (Biochem. J., **38**, 224, 1944). The results are shown in table 1.

TABLE 1

Amino acids in healthy Chlorella cells

AMINO ACIDS	RELATIVE AMOUNT
Glycine....	+
Alanine.	+++
Valine	+
Leucine ⎱ and ⎬......	+
Isoleucine ⎰	
Serine...	+
Tyrosine..	+
Proline...	+
Aspartic acid...	++
Glutamic acid..	+++++
Glutamine...	+
Asparagine....	+
β-Alanine...............	+

The full alcoholic extract of 13×10^8 cells was used.

It can be seen that alanine and glutamic and aspartic acids are present in larger proportions than the other amino acids found. This furnishes added evidence in support of a link between carbohydrate metabolism and protein formation comparable to that described in animal tissues (Evans: Ann. Rev. Biochem., **13**, 187, 1944).

Several investigators (Rose: Physiol. Rev., **18**, 109, 1938; Block and Bolling: The Amino Acid Composition of Proteins and Foods, Charles C. Thomas, 1945) have suggested a list of amino acids called indispensable as protein builders in animal tissues. These include arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine, and valine. Among them, only leucine, isoleucine, and valine were detected in the *Chlorella* hydrolyzate. It may be possible that the other essential amino acids were present in quantities too small to be identified by the method used. As there was no indication of their

presence, the results are interesting in that they tend to show the existence of plant proteins without several of the amino acids that have been demonstrated to be indispensable in animal tissues.

AN ENRICHED SEMISOLID MEDIUM FOR HUMAN STRAINS OF PLEUROPNEUMONIALIKE ORGANISMS

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Studies relating to human strains of pleuropneumonialike organisms are greatly handicapped by the very meager growth that can be obtained with recommended media. A number of combinations of available media were studied to determine whether these organisms could be induced to grow more luxuriantly. The following soft agar medium has given consistently good growth with available human strains:

Cystine	0.5 g
Trypticase	20.0 g
Agar	3.5 g
Sodium chloride	5.0 g
Sodium sulfite	0.5 g
Water	1 liter

All the foregoing ingredients are available in combined form (with 0.017 g phenol red) as "cystine trypticase agar" from the Baltimore Biological Laboratory. To the above is added 5 g yeast extract (Difco). The medium is then adjusted to pH 7.8 to 8.0 and is autoclaved at 12 pounds pressure for 15 minutes. When it is cool 250 ml sterile normal rabbit serum that has been heated at 56 C for 30 minutes is added.

After inoculation and 2 to 5 days' incubation at 35 C, floccular, slightly spreading growth is clearly visible with the naked eye. Stock cultures can be maintained in this medium for a month in the refrigerator, but storage at −20 C or −70 C is recommended.

IMMUNIZATION WITH HEAT-KILLED MYCOBACTERIUM PARATUBERCULOSIS IN MINERAL OIL

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In recent years a number of investigators have observed that antigens elicit a more powerful immunological response when suspended in mineral oil than when dissolved or suspended in water. (For references see Halbert and others, 1946). Paratuberculosis (Johne's disease) of sheep and cattle is a chronic mycobacterial infection against which a good vaccine would be of great value. We have tried to inoculate groups of sheep with heat-killed cultures of *Mycobacterium paratuberculosis* suspended in mineral oil. A report of two such experiments will be presented here.

Previous workers have injected *living* cultures of *M. paratuberculosis* suspended in mineral oil into cattle and have observed that allergy is produced, and in some cases, at least, a certain resistance to infection. Vallée and Rinjard in 1926 appear to have been the first workers to report this. (For references see Vallée and others, 1941). In many cases, however, the administration of living vaccine would not be safe under field conditions because of the danger of introducing the infection into new herds.

In the present experiments two groups of sheep were injected with varying quantities of dead *M. paratuberculosis*. Their serological response was checked by testing their sera for complement-fixing antibodies using antigens from infected intestinal tissue as previously described (Sigurdsson, 1945, 1946, 1947). The development of allergy as manifested by the cutaneous reaction to avian tuberculin was also noted. One injection of 0.1 ml of commercial avian tuberculin was used, and the reaction was read 48 hours after the injection.

A mixture of two old bovine strains of *M. paratuberculosis* was employed for the preparation of the vaccine. One of these strains was the so-called Teps strain, which was obtained from Dr. R. E. Glover of the National Institute for Medical Research, Farm Laboratories, Mill Hill, London. The other strain was called "strain 18" and was obtained from Dr. B. T. Simms of the Animal Disease Research Laboratory of the Bureau of Animal Industry, Auburn, Alabama. The bacteria were cultivated on a liquid medium of the following composition (originally described by Dunkin in 1933): potassium phosphate secondary 1.0 g, ferric citrate 0.06 g, magnesium sulfate 1.0 g, sodium citrate 0.5 g, glycerol 70 ml, dried *Mycobacterium phlei* 0.3 g, distilled water 1,000 ml.

When the cultures were fully grown (about 8 weeks old), they were harvested. In the first experiment the whole cultures were steamed at 100 C for 1 hour, and the bacteria were then filtered off and dried. In the second experiment, the bacteria were filtered off while alive, and samples of the wet culture were kept at different temperatures for 60 minutes in order to test their heat resistance. It

was found that 60 C for 1 hour would effectively kill the organisms. The bulk of the culture was then killed by being held at 70 C for 1 hour.

In both experiments the bacteria were next dried in a vacuum oven at about 40 to 45 C and were then suspended in sterile mineral oil containing 0.3 per cent phenol. The amount of bacteria suspended was so adjusted that the desired dose would be contained in 2 ml of the mineral oil. This vaccine was then inoculated subcutaneously on the inside of the right leg of 8- to 10-month-old lambs.

In the first experiment 9 lambs were employed. They were divided into 3 groups, one of which received 50 mg, another 100 mg, and the third 300 mg, of the dried culture. In the second experiment 10 lambs were used. This time they were divided into 5 groups of 2 lambs each, which received 5 mg, 10 mg, 20 mg, 50 mg, and 100 mg, respectively. As a control several of the lambs were also injected subcutaneously in the left leg with 2 ml of sterile mineral oil. The lambs were bled at monthly intervals, or more frequently in the beginning, and at the same time their cutaneous allergy to avian tuberculin was tested.

RESULTS

For the first few days after the injection no reactions were observed. Most of the animals developed a temperature of 40 C or more for 1 or more days between the third and twelfth days after the injection. None of them seemed seriously ill. All the sheep developed infiltrates of considerable size at the site of injection. These were first noticed on about the fifth day, and they increased relatively rapidly for the next 3 or 4 weeks, and then more slowly for a few weeks afterwards. When the two experiments were terminated after 20 and 17 months, respectively, the infiltrates had decreased, but according to our estimation, which was admittedly not very accurate, they were still about half the maximal size. In 6 of the 19, localized abscesses of considerable size were noticed at the site of injection, and in at least 2 cases these opened and emptied themselves. The lesions at the site of injection, although of considerable size, and of long duration, did not seem to cause the animals any trouble. At autopsy the infiltrates were found to be even larger than had been anticipated from their *in vivo* appearance.

When these experiments were terminated, the infiltrates were dissected out and carefully examined. They were composed of rather dense fibrous tissue with cavities of varying size filled with dry, light gray or greenish necrotic material. In most cases acid-fast bacteria were readily demonstrated, and their number had a definite correlation with the number of bacteria introduced at the beginning of the experiment. The preparations from the animals that had received the largest doses in the beginning showed a tremendous number of acid-fast bacteria comparable to those found in a heavily infected intestinal mucosa in Johne's disease. We tried to cultivate these bacteria from the lesions on Dunkin's medium but with negative results. We also attempted to cultivate bacteria from the lesions on ordinary bacteriological media. In one case only did a few colonies of staphylococci appear. The other infiltrates proved to be sterile.

The average weight of the infiltrates at the site of the injection in the 16 animals that came to autopsy was 114 grams, the smallest lesion being 22 grams and the

heaviest one 274 grams. Table 1, which is based on both the first and second experiments taken together, shows the average weight of the infiltrates that developed in the sheep after the various doses of vaccine given. There seems to be some tendency for heavier infiltrates in the groups that received the larger inocula.

The lesions may be conveniently divided into three groups according to their degree of infiltration into the underlying tissue. In the first group the lesion was confined to the subcutaneous connective tissue and did not infiltrate the muscle at all. These lesions could be easily peeled from the superficial fascia. Seven of

TABLE 1

The weight of infiltrates found at autopsy correlated with the weight of dried bacteria originally injected

	WEIGHT OF DRIED BACTERIA INJECTED					
	5 mg	10 mg	20 mg	50 mg	100 mg	300 mg
Average weight of infiltrates in each group of sheep..............	62 g	61 g	77 g	147 g	99 g	202 g

TABLE 2

Type of lesion in the different animals related to dose of vaccine injected

FIRST TYPE, LESION NOT ADHERENT		SECOND TYPE, LESION ADHERENT TO MUSCLE		THIRD TYPE, LESION INFILTRATES DEEPLY	
Sheep no.	Mg injected	Sheep no.	Mg injected	Sheep no.	Mg injected
36	5	33	10	21	50
38	5	32	20	34	50
35	10	37	20	24	100
20	50	28	300	30	100
23	100			27	300
25	100				
29	100				
Mean dose .	52.9		87.5		120.0

the animals belonged to this group. The second type of lesion infiltrated the fascia and the superficial muscle but did not penetrate deeply. These lesions could not be removed without removing considerable portions of the muscle at the same time. Four animals came into this group. In the third type the infiltrates penetrated deep into the muscle and followed the lymph path under the inguinal ligament to the internal iliac lymph glands. These infiltrates were very extensive and difficult to dissect out. Five sheep belonged to this group. Table 2 shows the relationship between the size of the injected dose of vaccine and the type of lesion. It seems that the larger doses tended to produce the more severe kinds of lesions.

The internal iliac lymph glands were found to be enlarged in most instances,

and in one instance they weighed as much as 70 grams. They were in all cases examined microscopically for the presence of acid-fast bacteria, but these were never found. Allergy toward the avian tuberculin injected intracutaneously was observed in all the animals. In three cases this lasted for 2 months or less. In the remaining cases it lasted for 7 months or more.

Complement-fixing antibodies reacting with the tissue antigen described in previous papers (Sigurdsson 1945, 1946, 1947) were present in very high titer in every case. This antigen is extracted by a special method from the intestinal mucosa of sheep infected with *M. paratuberculosis*. The antibody titers in these vaccinated animals were much higher than those found in natural cases of the disease. When the strength of the reaction is expressed with a numerical value (Sigurdsson, 1945), a strong reaction in a natural case of the disease will give a value of 6 to 10, but in these experimental sheep the value would lie around 20. These values indicate that, under the condition of our complement fixation test, about 0.0625 to 0.0156 ml of serum from a strong natural case would give a complete fixation of complement (Sigurdsson, 1945), but only about 0.005 ml, or about 32 times to 128 times less, of the serum from our experimental sheep would be required to produce the same effect. The technique of the complement fixation tests as carried out in our laboratory has been described earlier (Sigurdsson, 1945).

In the present experiments the serum samples were collected and tested over a period of 20 months. It was therefore necessary in each test to include a standard control so that the results for the different samples would be strictly comparable. To this end samples of serum from the nine animals in the first experiment collected after their antibody titer had risen to a high level were kept frozen and tested several times. A standard value for them was thus experimentally obtained. The sera were then included as standard in every test on the fresh sera from the experimental sheep. If the values obtained on any particular day for these standard sera were other than those originally fixed for them, they were corrected to the original values and all the results for the other sera tested on the same day were then adjusted correspondingly. This should have secured reasonably comparable results over the whole experimental period.

Figure 1, in which the average numerical values are plotted against time, shows graphically the outcome of the complement fixation tests carried out on the 19 sheep during the experimental period. The results from the two experiments are shown separately. (On the ordinate are plotted the average numerical values [Sigurdsson, 1945] for the sera on any given day). In the first experiment the value was practically zero (0.17) on the day of injection, and in the second experiment it was zero. The titer rose very steeply in the beginning and continued to rise for the first 3 or 4 months. After that it fell somewhat but very slowly. When the two experiments were terminated after 20 months and 17 months, the titer of complement-fixing antibodies as measured in our test was still extremely high.

A possible source of error is the possibility that the "standard sera" may have

gradually lost in strength during the experimental period. This cannot be excluded. If this did happen, it would mean that the later values in the graph are too high. This error, however, could hardly be of much importance as the "corrections" during the latter part of the experimental period did not tend to raise the outcome of the tests significantly.

The data on the individual animals cannot be published *in extenso* as this would require too much space, but it should be stated that the reactions of the individual animals were remarkably uniform in that they all reached a comparable level of antibody production. We were unable to detect any individual variation in the strength of the serological reaction that could be traced back to
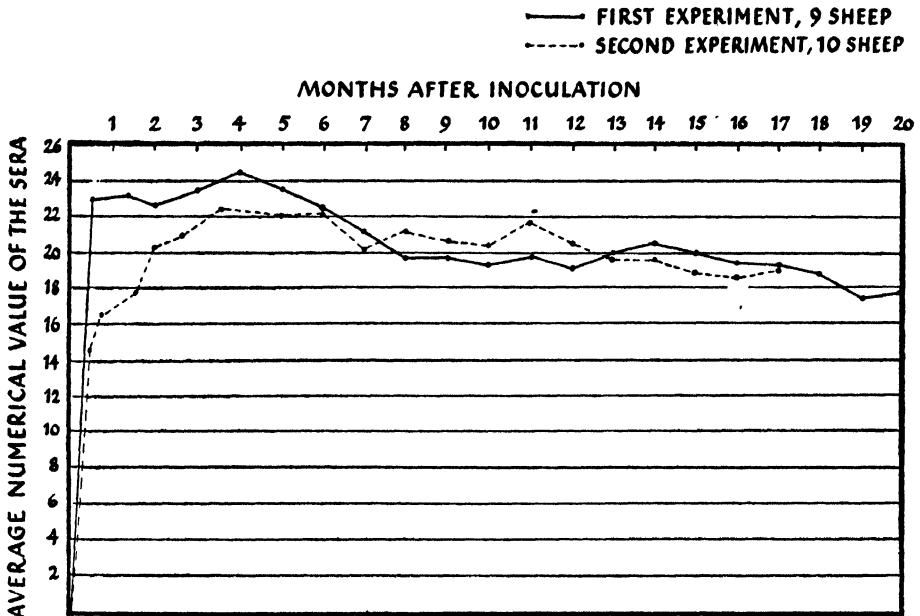
Figure 1. Average strength of serological reaction in the two experiments.

variations in the size of the injected dose of bacteria, although this dose varied from 5 milligrams to 300 milligrams.

As already stated the bacteria were killed at 100 C in the first experiment and at 70 C in the second experiment. As far as could be seen this difference also was without influence on the outcome of the serological reactions. These two facts seem to justify our treating the serological reactions *en bloc* as done in the graph. The same was true of the intradermic tuberculin test. This did not seem to be influenced by the number of bacteria originally injected nor by the temperature at which they had been killed.

It would obviously be of interest to learn how animals that have been injected once with this "vaccine" would react to a second injection of the same preparation. When the two experiments had lasted, one for 15 and the other for 12 months,

this trial was made by dividing the group in each experiment in half and inject-
ing one-half with 50 milligrams of heat-killed bacteria (70 C for 1 hour) suspended
in sterile mineral oil. The injection was given subcutaneously on the inside of
the left leg. When the animals were divided for this purpose, care was taken that
the different measurable qualities of the sheep, such as body weight and strength
of serological response, should be divided equally between the two new groups.
Three animals which had not been injected previously were injected as controls.

The temperature of all these sheep was taken for some days after this injec-
tion, and the average of the temperature for the three groups is reproduced in
figure 2. If temperatures below 40 C are considered normal for sheep, the rein-
jected group showed a rather insignificant rise in temperature, whereas the group
that was injected for the first time showed a considerable and prolonged rise.
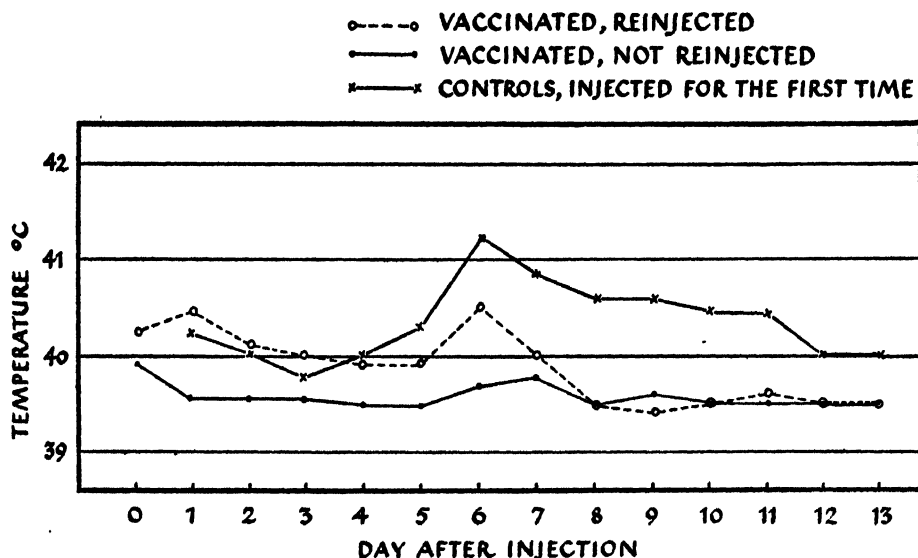
Figure 2.

The sheep that were injected for the second time developed large infiltrates at
the site of the new injection. These were comparable in size to those developed
after the first injection, and they appeared after about the same time and in-
creased at about the same rate as did the infiltrates in the control animals that
had not been injected before.

At autopsy the average weight of the infiltrates that developed at the site of
the second injection in the reinjected animals was 115 grams, and these infiltrates
were found to penetrate the underlying tissue to about the same extent as did
the original infiltrates. The second injections had no detectable influence on the
sheep concerned nor on their sensitivity to the avian tuberculin. A glance at
figure 1 will show that the average curves were not affected by the injections,
which in the case of the first experiment took place in the fifteenth month and

in the case of the second experiment in the twelfth month after the first injection.

DISCUSSION AND SUMMARY

The subcutaneous injection into sheep of heat-killed *Mycobacterium paratuberculosis* (*bovis*) suspended in mineral oil elicited a powerful and protracted serological response.

Two sets of experimental results were obtained, one with bacteria that had been killed by being boiled for 1 hour and the other with bacteria which had been killed by being held at 70 C for 1 hour. Before injection all the bacteria were dried to a constant weight in a vacuum oven at about 40 to 45 C. The individual dose varied from 5 milligrams to 300 milligrams and was suspended in 2 ml of sterile mineral oil containing 0.3 per cent phenol.

The results indicate that all the different doses elicited a maximal serological and allergic response since the antibody titers and allergy produced by 5 milligrams of bacilli were just as high as those produced by the larger doses.

From this it appears that 5 milligrams of dried bacilli contain so much of the antigen or antigens responsible for these reactions that no further effect is obtained by increasing the dose. It is interesting in this connection that at autopsy, 20 months after the injection, a large number of bacteria were found morphologically intact in the inoculum in the cases where large doses had been used, although the bacteria had been killed by boiling before they were injected.

The infiltrates at the site of inoculation grew to rather considerable proportions and showed very little tendency to regress during the experimental period (20 and 17 months). In some cases they infiltrated deep into the underlying muscle, but it seems that this tendency to penetrate was less pronounced in the animals that received the smaller doses.

The infiltrates did not seem to cause the animals any trouble, which fact was rather surprising in view of their size.

The difference in the temperatures at which the bacteria were killed was not seen to influence the experimental results.

Half of the sheep employed in the first experiment were reinoculated 15 months after the first injection, and half of the sheep in the second experiment 12 months after the first injection. These reinoculated sheep developed infiltrates indistinguishable from the original ones, but the febrile reaction observed after the second injection was much less pronounced than after the first injection.

The second injection did not noticeably influence the level of complement-fixing antibodies nor the allergic state in these experimental sheep.

The possibility that injections of this "vaccine" will increase resistance to natural injections is now being investigated.

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THE EFFECT OF ORALLY ADMINISTERED STREPTOMYCIN AND SULFATHALIDINE UPON THE BACTERIAL FLORA OF THE COLON

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The oral administration of antibacterial agents is commonly employed in the preparation of patients for surgery of the large bowel. Largely as the result of investigations by Poth (1943, 1946) and Poth and Ross (1944) sulfasuxidine (succinylsulfathiazole) and sulfathalidine (phthalylsulfathiazole) are recognized as effective drugs for this purpose in that they bring about and maintain a significant reduction in the coliform count of the colon. However, these nonabsorbable sulfonamides require from 3 to 10 days to produce a significant reduction (99.99 per cent) and have no appreciable effect upon enterococci, *Salmonella*, *Pseudomonas*, or *Proteus* organisms (Firor, 1942).

Streptomycin acts more rapidly than the sulfonamides and has a different antibacterial spectrum. The alterations in intestinal flora of human beings resulting from oral administration of this drug were determined by Zintel *et al.* (1947), who found streptomycin to be more efficient than sulfasuxidine since it produced a more rapid decrease in coliform organisms and caused the virtual disappearance of enterococci. Although the development of streptomycin resistance was not reported by these investigators, the likelihood that this factor would eventually limit its usefulness was obvious. Therefore, we undertook a study of streptomycin and sulfathalidine in combination. The clinical and bacteriological results of this study (Rowe *et al.*, 1948) indicated that the combination produced a more rapid reduction in coliform count than either one alone, but that it did not prevent the appearance of streptomycin-resistant organisms in large numbers.

At the present time the advisability of administering streptomycin orally before an operative procedure upon the large bowel is a controversial subject. Zintel *et al.* (1947), Morton and Smith (1948), and Herfort and Stoddard (1948) report favorable clinical results. On the other hand, Poth and co-workers (1948) and Lockwood *et al.* (1949) believe that there is no increase in antiseptic action when streptomycin is combined with sulfathalidine. Furthermore, Lockwood and his group (1949) consider the preoperative use of this antibiotic as dangerous because of the risk of the development of resistance and the establishment of a highly resistant flora at the time of operation.

The differences recorded by the authors just cited appear to be due in part

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[1] Part of the material in this paper also appears in a thesis submitted to the Temple University School of Medicine in partial fulfillment of the requirements for the degree of Master of Science (Proctology).

to a lack of uniformity in the cultural methods used, particularly to failure to employ an inactivator of streptomycin, and in the length of time the drugs were administered. Therefore, it is the purpose of this paper to describe a practical method for counting viable coliform bacilli in the feces of patients receiving streptomycin by mouth and to describe the effects observed by us when sulfathalidine and streptomycin are administered in combination. Certain observations upon the susceptibility of organisms isolated before and during administration of the drugs are also reported.

METHODS

Coliform Counts

Our technique was patterned after that of Poth (1946) in that a small quantity of the fecal specimen was added to diluting fluid and appropriate dilutions were streaked to agar plates. Following incubation the typical coliform colonies were counted and these figures converted to the number of viable coliform bacilli per g of wet feces.

Fecal specimens were classified as (a) formed, (b) semiformed, or (c) fluid. A biconvex 4-mm loopful of formed or semiformed stool was found to weigh from 20 to 22 mg; the figure of 20 mg was used. Fluid specimens weighed approximately 16 to 17 mg per 4-mm loopful. Thus, the weight factors for converting colony counts to the number of bacteria per g were 1:50 and 1:60, respectively.

Preliminary specimens. One 4-mm loopful of feces was emulsified in 2 ml of distilled water and the mixture permitted to stand until large particles, if present, had settled out. This tube represented approximately a 1:100 dilution of the specimen. Further dilutions were made in a similar manner. One 4-mm loopful of diluted material (approximately 0.02 ml) was placed on the center of a Levine eosin methylene blue agar (Difco) plate and thoroughly spread over the surface with a wire loop so as to get even distribution of growth. After 24 hours' incubation at 37 C the typical coliform colonies were counted and the number of viable coliform bacilli per g of fecal material was calculated. As far as possible, all counts were based upon the average values of plates containing from 30 to 300 colonies.

Specimens containing sulfathalidine. Specimens were emulsified and diluted in 5 mg per cent aqueous *para*-aminobenzoic acid (PABA). It was not necessary to allow the fecal sulfonamide inoculum and PABA to remain in prolonged contact in the tubes. Dilutions lower than 1:100 were prepared by adding the same inoculum to 1.0 ml of PABA solution (1:50) or to 0.5 ml (1:25). The absence of colonies on the plate streaked from a 1:25 dilution tube was recorded as less than 1:1,250 (or 1:1,500 in the case of fluid stool), which constituted the baseline count. It was not necessary to incorporate PABA in the plating medium.

Specimens containing streptomycin. Semicarbazide hydrochloride (Eastman-Kodak) was found to be a satisfactory inactivator of streptomycin. A fresh 6 per cent solution was made weekly, sterilized by Seitz filtration, and stored in the refrigerator. Because this solution is very acid, it must be neutralized at the time of use (Rake and Donovick, 1946). We employed sodium acetate as a 5 per cent

aqueous solution, sterilized by autoclaving at 10 pounds pressure for 10 minutes, and stored at room temperature.

The 1:100 dilutions of feces were prepared in tubes containing 0.1 ml of the stock semicarbazide solution, 0.1 ml of the stock sodium acetate solution, and 1.8 ml of water. Higher dilutions were prepared in sterile water, lower dilutions by reducing the volume of water. Thus, a 1:25 dilution tube contained 0.3 ml of water with a final concentration of 1.2 per cent semicarbazide and 1.0 per cent acetate. Neither agent was incorporated in the EMB agar plates.

Two types of preliminary experiments were carried out to determine whether the semicarbazide-acetate solution effectively neutralized streptomycin and was free of bacteriostatic action under test conditions. One loopful of a saline suspension of *Escherichia coli* susceptible to 1 μg per ml was added to a tube containing 500 μg of streptomycin per ml, 0.3 per cent semicarbazide, and 0.25 per cent acetate. After contact for 20 minutes one loopful was streaked to EMB agar. At the same time a second plate was streaked from a control suspension in water. The plate counts were essentially the same.

The second type of preliminary test compared colony counts obtained from fecal specimens diluted in semicarbazide solution and in water. The results below represent the range of variation noted and demonstrate the absence of any significant degree of inhibition.

Specimen	Dilution fluid	Coliforms per gram
No. 1	Water	111,750,000
No. 1	Semicarbazide-acetate	109,000,000
No. 2	Water	186,500,000
No. 2	Semicarbazide-acetate	154,250,000

The minimum time required for preliminary contact of streptomycin and semicarbazide was determined by the filter-paper-disk method for testing antibiotic susceptibility described by Bondi et al. (1947). EMB agar plates were heavily inoculated (0.3 ml) with a suspension of *Escherichia coli*, susceptible to 2 μg of streptomycin per ml, which was spread evenly over the surface. Whatman no. 2 filter paper disks of 6.5-mm diameter were dipped into freshly prepared 0.3 per cent semicarbazide, 0.25 per cent acetate solution before and at various intervals of time after the addition of streptomycin in a final concentration of 1,320 μg per ml. The disks were then placed on the freshly inoculated agar plates and the zones of inhibition measured after 24 hours' incubation. The values that appear in table 1 are representative.

It is apparent that preliminary contact with semicarbazide for 15 minutes is sufficient to inactivate streptomycin under the test conditions. It should be noted, however, that the actual time required for neutralization was not determined by this procedure since the two agents remained in contact on the disks during the entire period of incubation. Repeat tests with this solution produced similar results up to 7 days, after which time inactivation of streptomycin gradually decreased. In our experiments fresh solutions were prepared every 7 days and left in contact with the fecal specimen for 10 to 20 minutes.

The streptomycin content of feces from patients who received orally 2 g daily at 4-hour intervals was also determined. One loopful of stool was diluted in tubes containing 1 ml of sterile distilled water. Disks were immersed in these tubes and placed on the surface of meat extract agar plates, pH 7.6, previously seeded with the Smith strain of staphylococcus. The zones of inhibition after 24 hours' incubation were measured and interpreted by comparison with a standard curve obtained at the same time.

Assays of 80 specimens from 10 individuals produced values ranging from 240 to 12,000 μg of streptomycin per g of feces. It had already been determined that a final concentration of 0.3 per cent semicarbazide inactivated a 1,320 μg per ml solution of streptomycin. Since the lowest dilution of feces to be made in 0.3 per cent semicarbazide solution was 1:25, the greatest streptomycin concentra-

TABLE 1

Inactivation of streptomycin by semicarbazide

DISK IMMERSED IN	PERIOD OF CONTACT	DIA. OF INHIBITION ZONE (mm) (Disk 6.5 mm)
Sc-Ac, pH 4.0		7.0
Sc-Ac, pH, 6.5*		None
SM (1320 μg/ml)		16.0
SM + Sc-Ac, pH 5.0	5 sec	8.0 (incomplete)
SM + Sc-Ac, pH 5.0	5 min	7.5 (incomplete)
SM + Sc-Ac, pH 5.0	10 min	7.5 (incomplete)
SM + Sc-Ac, pH 5.0	15 min	None
SM + Sc-Ac, pH 5.0	20 min	None

Sc-Ac, semicarbazide-acetate solution. SM, streptomycin (1,320 μg per ml).
* Adjusted with NaOH.

tion requiring inactivation would be 480 μg per ml. Thus, the procedure as described provided for the inactivation of more than 2.5 times the highest concentration of streptomycin likely to be encountered.

"Total" Counts

This procedure was used only with specimens from certain patients receiving both drugs. One loopful of feces was suspended in 10 ml of 0.3 per cent semicarbazide solution and further dilutions were made in water. One-ml volumes were added to melted veal infusion agar containing 5 mg per cent PABA and poured agar plates were prepared. Each dilution tube was plated in duplicate, one plate being incubated in the usual manner, the other anaerobically according to the method of Spaulding and Goode (1939). After 48 hours' incubation the colonies were counted and recorded separately as aerobic and anaerobic counts. The term "total" count is used with qualification since the medium employed did not permit appreciable growth of certain bacterial genera, notably the lactobacilli.

RESULTS

The subjects in this study were patients with carcinoma of the large bowel who were being prepared for surgery. The attempts that were made to standardize various factors known to influence the enteric bacterial flora have already been described (Rowe et al., 1948). The subjects were divided into three groups: the first received sulfathalidine, 0.1 g per kilo daily; the second, 2 g of streptomycin daily; the third, a combination of the two drugs in dull dosage. In all instances medication was given at 4-hour intervals.

Sulfathalidine coliform counts. Data were obtained on 7 patients receiving the drug for a period of 4 to 11 days. The number of specimens per patient ranged from 8 to 20. In all instances the viable coliform count decreased to 0.01 per

TABLE 2

Results of coliform and "total" counts from 36 patients

GROUP	TOTAL	NO RE- SPONSE	DECREASE TO		AVG TIME (DAYS) FOR DECREASE TO		REVERSION AFTER DECREASE TO 0.01%
			0.1%	0.01%	0 1%	0.01%	
Coliform							
Sulfathalidine, 0.1 g/kilo/day.	7		7	7	3.1	3.5	None
Streptomycin, 2 g/day.......	11	2	9	9	1.0	1.9	None
Streptomycin, 0.5 g/day.....	6	2	4	2	3.7	4.9	2 (2½ and 8 days)
Streptomycin, 2 g/day + sul- fathalidine, 0.1 g/kilo/ day.	12		12	12	0.5	1.1	2 (5 and 9 days)
"Total"							
Aerobic	6	3	3	2	1.0	2.1	1 (3 days)
Anaerobic..	6	4	2	1	1.7	2.0	1 (4 days)

cent of the preliminary count. The time required to produce this degree of reduction ranged from 1 to 10.5 days with an average of 3.5 (table 2). Reversion in count, following an initial significant decrease, was not observed in this group.

Streptomycin coliform counts. Results are available on 11 patients. Four to 11 specimens were collected from each individual during periods ranging from 2½ to 5 days. In 9 of the 11 individuals the decrease was rapid, a reduction to 0.01 per cent being reached in the average time of 1.9 days. At 24 hours the decrease amounted to 99.9 per cent (table 2).

In the other two instances, however, the results were very different in that the counts remained high during the entire period of observation. Cultures obtained both from preliminary specimens and from others collected during drug administration were tested for streptomycin sensitivity by the disk method of Bondi et al. (1947). The predominating organism in a preliminary specimen from the first individual was *Aerobacter aerogenes* with a sensitivity of approximately

1 μg per ml. But the culture collected 2½ days after administration of streptomycin contained almost a pure culture of highly resistant *Escherichia coli*. In the second instance the predominant organism originally was *E. coli* susceptible to 1 μg, but 3 days later this had been replaced by a highly resistant *E. coli*. Thus, the daily administration of 2 g of streptomycin produced a significant reduction in fecal coliform bacilli within 2 days in 9 of 11 individuals but had no appreciable effect in the other 2.

Early in this study 6 patients were placed on a daily dosage schedule of 0.5 g of streptomycin. The number of specimens per patient varied from 6 to 20 and extended over periods ranging from 5 to 8 days. There was no appreciable effect upon coliform bacilli in 2 instances, a partial delayed decrease in 2, and a rapid significant decrease followed by reversal in count in the remaining 2 persons. Because of the erratic results observed, this program was discarded as inadequate.

Combined sulfathalidine-streptomycin coliform counts. Satisfactory data were obtained from 12 patients given a combination of 2 g of streptomycin and 0.1 g per kilo of sulfathalidine daily. The periods of observation ranged from 27 hours to 10 days; from 6 to 33 specimens were examined from each individual. In most instances there was a precipitous drop to the base-line level of less than 1,250 per gram. The average values for these 11 individuals showed a decrease to 0.1 per cent at 12 hours and to 0.01 per cent by 1.1 days (table 2).

The coliform count showed a definite reversion in two instances and a questionable rise in another. In the first, the increase did not begin until the ninth day when *Aerobacter aerogenes* appeared, which was highly resistant to both drugs. The number increased rapidly and attained counts approximating the original level. In the other individual reversion began on the fifth day and was also attributable to *Aerobacter aerogenes*. A highly susceptible intermediate type of coliform predominant in a preliminary specimen was not recovered at this time; instead, the feces contained pure cultures of *A. aerogenes* highly resistant to streptomycin and to sulfathalidine. This patient was operated on the ninth day. Contamination occurred during operation and resulted in peritonitis, multiple abscess formation, and death. The pure cultures of *A. aerogenes* obtained at autopsy were resistant to 1,000 μg of streptomycin per ml and to 200 mg per cent sulfathalidine.

These results suggest that combined administration of sulfathalidine and streptomycin produces a more rapid decrease in fecal coliform bacilli than either drug by itself. It is apparent, however, that the development of resistance cannot be prevented by this procedure.

"Total" counts. The coliform count has been used by Poth (1946) and others as the principal criterion for judging the effect of sulfonamides upon the fecal bacterial population. However, the data of Zintel *et al.* (1947) and of Lockwood and his associates (1949) show that a decrease in viable coliform organisms is not associated with a similar drop in enterococci or clostridia. In order to test this point further both coliform and "total" counts were carried out with the specimens from 6 of the patients in the streptomycin-sulfathalidine group. The periods of observation ranged from 27 hours to 10 days and the number of speci-

mens per patient from 5 to 33. The average coliform and aerobic and anaerobic "total" counts appear in figure 1. It will be seen that the curves are quite similar.
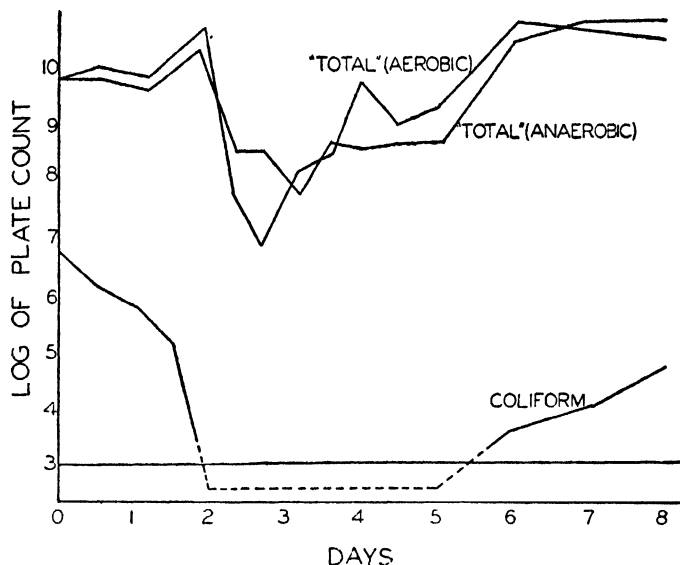
Figure 1. Average fecal coliform and "total" counts from six individuals receiving ora streptomycin and sulfathalidine.
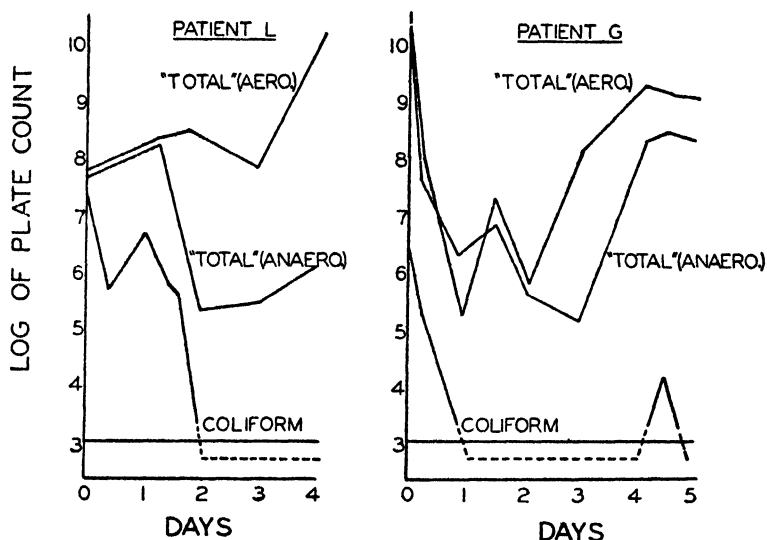
Figure 2. Fecal coliform and "total" counts from two individuals receiving streptomycin and sulfathalidine. The reversion in patient L was due to *Alcaligenes faecalis,* enterococci, and yeasts, and in patient G to enterococci and *Pseudomonas aeruginosa.*

However, when the results from each individual are examined separately, significant variations become evident (figure 2). Only 2 of the 6 patients showed a significant decrease (99.99 per cent) in the aerobic "total" count, and only one

in the anaerobic count. Two of the counts reverted in 3 to 4 days owing to the multiplication of enterococci and *Pseudomonas* organisms.

Definite conclusions cannot be drawn from a study of 6 patients. Nevertheless, the coliform count with one exception did reflect the "total" counts for a period up to 2 days. After this time the two types of counts diverged sharply and in no individual case could the number of coliform bacilli be taken as a reliable index of the total population. Since coliform bacilli constitute only one of the many groups of enteric organisms, it is not surprising that they will only occasionally be responsible for or participate in "total" count reversions. All 6 individuals examined by us showed either "total" count reversions or lack of a significant decrease. In only one instance were coliform bacilli responsible for the failure; the others were caused by enterococci, yeasts, *Alcaligenes faecalis*, *Pseudomonas aeruginosa* and related organisms, and members of the *Bacteroides* group.

Studies on drug susceptibility. It had been our hope that a combination of the two drugs might cause a consistent and permanent decrease in coliform bacilli. Since development of resistance did occur, studies were carried out to determine the susceptibility of organisms recovered from cultures obtained before and during drug administration. Selected specimens, when received, were subcultured directly to agar slants, incubated for 24 hours, and preserved in the cold. At the time of testing they were plated on EMB agar and incubated, and several coliform colonies were inoculated into a single tube of broth. The approximate susceptibility to streptomycin of the 18- to 20-hour cultures was determined by the disk method and to sulfathalidine by adding a light inoculum to tryptone broth.

Satisfactory isolations were made from 31 specimens originating from 13 different patients, 4 of whom were in the 0.5-gram-streptomycin group, 2 in the 2-gram-streptomycin group, and 7 in the combined sulfathalidine-streptomycin group. Table 3 contains representative data on strains isolated from patients showing persistence of coliform organisms. In patients K, D, and H, the original predominant organism disappeared and was replaced by a different type resistant to streptomycin, and in the case of patient H to streptomycin and also to 200 mg per cent sulfathalidine. In patient G, a resistant type of *Escherichia coli* appeared which, however, was apparently inhibited by sulfathalidine, the *in vitro* susceptibility of which was 12.5 mg per cent. Drug-resistant coliforms were not recovered from patient M, who showed temporary rises on the fifth and seventh days. It is of interest that the streptomycin concentration in specimens nos. 9 and 14 from patient H was respectively 1,600 and 1,200 μg per g, and in specimens nos. 10 and 15 from patient M it was 12,000 μg.

The lack of uniform response to combined sulfathalidine-streptomycin administration was disappointing. Since present evidence indicates that these drugs have different antibacterial mechanisms, it might be expected that coliform bacilli resistant to one drug would be inhibited by the other, as in the case of patient G, and that those resistant to both compounds would rarely be encountered. However, a multiplication of streptomycin-resistant, sulfathalidine-susceptible organisms was observed on several occasions in spite of the fact that sulfathalidine was being administered at the time. The most likely explanation for this dis-

crepancy is the slow action of the sulfonamide, since the number of such variants was seen to level off and decline over a period of 1 to 3 days. The development of resistance to streptomycin was encountered frequently. Thalidine resistance, however, was uncommon, and resistance to both drugs was noted only twice.

It seemed of interest to determine the *in vitro* bacteriostatic action of the two drugs in combination upon bacteria resistant to one or both agents. Fourteen strains, including coliform bacilli, viridans streptococci, and enterococci were selected. These originated from 4 individuals in the combination prophylaxis

TABLE 3

Streptomycin susceptibility of coliform bacilli isolated before and during drug administration

GROUP	PATIENT	SPECIMEN	DATE	COLIFORM COUNT	STREPTOMYCIN SUSCEPTIBILITY (µg/ml)	PREDOMINATING ORGANISM
SM, 0.5 g/day	B	Prelim.	3/12	14×10^6	2	*E. coli*
	B	Prelim.	3/13	3×10^6	2	*E. coli*
	B	No. 6	3/18	3750	4	*E. coli*
	K	Prelim.	3/29	11×10^6	4	*E. coli*
	K	No. 4	4/6	158×10^6	Resistant	*A. aerogenes*
SM, 2 g/day	D	Prelim.	3/4	250,000	2	*A. aerogenes*
	D	No. 4	3/9	20×10^6	Resistant	Coliform, intermediate
SM, 2 g/day + ST, 0.1 g/kilo/day	H	Prelim.	4/22	24×10^6	2	Coliform, intermediate
	H	No. 9	4/26	7,500	Resistant	*A. aerogenes*
	H	No. 14	4/27	1.6×10^6	Resistant	*A. aerogenes*
	G	Prelim.	7/8	1×10^6	0.5	*E. coli*
	G	No. 15	7/14	19,500	Resistant	*E. coli*
	G	No. 17	7/14	1,250	Resistant	*E. coli*
	M	Prelim.	4/17	24×10^6	4	*E. coli*
	M	No. 10	4/22	42,500	13	*E. coli*
	M	No. 15	4/24	67,500	13	*E. coli*

SM, streptomycin; ST, sulfathalidine.

group, 2 showing a good response, 1 producing reversion in count, and 1 failing to show any decrease. The results of these experiments can be summarized as follows: The activity of sulfathalidine and streptomycin in combination was somewhat greater than that of either one alone providing the test organism was susceptible to either one of the agents, but not when it was resistant to both.

DISCUSSION

No significant reversion in coliform counts was noted in patients receiving sulfathalidine for periods as long as 11 days, indicating that resistance to this drug is acquired slowly. Although no reversion occurred in the 2-g streptomycin group, it should be noted that the periods of observation did not extend beyond 5 days.

Thus, there was little opportunity for the multiplication of resistant forms. The inadequacy of streptomycin is further demonstrated by its failure to produce a significant decrease in 2 of 11 individuals.

Because bacteria readily develop resistance to streptomycin even in the presence of sulfathalidine, oral preoperative administration of this drug for more than 3 days would appear to be dangerous. The disastrous results that may follow prolonged streptomycin administration under these conditions was illustrated by patient H in our series, who harbored large numbers of resistant *A. aerogenes* at the time of operation and succumbed to postoperative infection with this organism.

On the other hand, streptomycin has the advantage of rapid activity and of reducing the enterococcus population. Therefore, a program that includes only brief administration of streptomycin may have merit. Two of the present authors (H.E.B. and R.J.R.) employ with apparent success a regimen of sulfathalidine for 7 days with the addition of streptomycin 2 days before operation. Theoretically this program should reduce the incidence of streptomycin resistance since relatively few organisms would be present at the time streptomycin is administered.

The combination of sulfathalidine and streptomycin used in these studies does not appear, however, to constitute a completely satisfactory procedure for preoperative antisepsis of the intestine. Studies along similar lines with bacitracin, aureomycin, or chloromycetin are indicated.

SUMMARY

A simple method is described for making coliform and "total" counts of fecal specimens from individuals receiving streptomycin by mouth.

Thirty-six patients being prepared for large bowel surgery were divided into three oral medication groups as follows: (1) sulfathalidine, 0.1 g per kilo per day; (2) streptomycin, 2 g daily; (3) a combination of the 2 drugs in full dosage. The time required to bring about 99.99 per cent reduction in coliform bacilli was 3.5 days, 1.9 days, and 1.1 days respectively. There were two failures in the streptomycin group.

Subsequent reversion in coliform count, indicating the development of resistance, was not observed in the sulfathalidine group over an average observation period of 6 days, or in the streptomycin group observed for an average of 3 days. But reversion did occur in 2 of 12 individuals who received both drugs for an average period of 6 days.

The oral administration of a combination of sulfathalidine and streptomycin had an irregular and temporary effect upon the "total" counts. The coliform count does not appear to be an adequate index of the effect of these drugs upon the total bacterial population.

Bacteria resistant to one of the two drugs were occasionally observed to disappear from the bowel because they were susceptible to the other.

Since bacteria rapidly develop resistance to streptomycin, even in the presence of sulfathalidine, it does not appear advisable to administer this drug for more

than 2 days before an operative procedure upon the large bowel. The serious consequences resulting from prolonged administration are illustrated by one case.

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STUDIES ON BACTERIAL VARIATION AND SELECTIVE ENVIRONMENTS

I. The Nature of the Selective Serum Factor Affecting the Variation of Brucella abortus

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Previously presented data (Braun, 1946b) proved the existence of a heat-stable, filtrable factor in the normal serum or plasma of cows, man, rabbits, and guinea pigs which selectively suppresses the establishment of nonsmooth *Brucella abortus* variants. The activity of this factor was clearly demonstrated in beef extract broth cultures containing 2 per cent normal serum. After inoculation with a smooth clone these cultures failed to show any variant types (dissociation) after 10 days of growth at 37 C, whereas after the same period of growth control cultures without serum consistently showed between 20 and 30 per cent rough and mucoid types in the originally smooth population. It was also shown that the growth rates of smooth types were not affected by the addition of serum in amounts that proved to be sufficient for this suppression of nonsmooth types.

In order to identify the factor responsible for this selective suppression of nonsmooth types the effect of various plasma fractions upon dissociation[2] of *Brucella abortus* was investigated. The results of these studies are reported in this paper.

MATERIAL AND METHODS

Bovine plasma fractions were kindly supplied by Armour and Company, and human plasma fractions were obtained from the Cutter Laboratories. These fractions were dissolved in distilled water or normal saline; the solutions were Seitz-filtered and added in various amounts to tubes containing 5 ml of broth. Unless otherwise noted, beef extract broth, buffered at pH 6.8, was used. Each tube was then inoculated with 200 million organisms from a smooth clone, isolated from *Brucella abortus*, strain 19. One of the two clones used in this work consistently showed 15 per cent and the other approximately 30 per cent dissociation, when samples were plated and counted after 10 days of growth in ordinary buffered beef extract broth. The dissociation index (i.e., the percentage

[2] As previously stated (cf. Braun, 1947) the use of the conventional term "dissociation" may be justified if applied to population phenomena, i.e., the spontaneous change or mutation of one or more members of a bacterial population and their subsequent establishment. In accordance with this definition, the use of the terms "dissociation index" and "dissociation" has been retained in this paper.

of dissociation after 10 days of growth in broth) was determined by counting and classifying at least 100 colonies on each plate (for further details of general procedures see Braun, 1946a).

The maximum concentration at which the fractions were tested varied because of the different solubilities of the various fractions employed. In no instance were more than 2 ml of dissolved fractions added to each culture, and in general the concentrations of the dissolved fractions were adjusted in such a manner that 0.1 ml, 0.2 ml, or 0.5 ml were added. Corresponding amounts of saline were added to the controls.

<div align="center">EXPERIMENTAL RESULTS</div>

Table 1 summarizes the results that were obtained after the addition of various concentrations of bovine plasma fractions. In this table the final concentrations of the respective fractions in individual cultures have been expressed as the percentage of the fractions' concentration in normal plasma. (All figures tabulated above the heavy line represent concentrations higher than those found in normal plasma and are therefore of little biological value.) It can be seen that the controls without supplement showed 30 to 34 per cent dissociation. Dissociation is suppressed in the presence of fraction II, when it is added to cultures in amounts that would correspond to 18 per cent plasma or more. Nonsmooth types are also selectively suppressed in the presence of fraction III-0, after the addition of amounts corresponding to 9 per cent plasma or more. A similar suppression of nonsmooth types is also produced in the presence of certain concentrations of fraction III-1. A significant but not complete suppression is also produced in the presence of fraction III-2. As illustrated in table 2, these "active" fractions contain either γ-globulin (fraction II) or a high percentage of β-globulin (fractions III-0, III-1, and III-2). No suppression of dissociation was produced in the presence of fractions I, IV, IV-3,4, or V (for their composition see table 2); in fact, both fractions I and V enhanced the percentage of dissociation. Two subfractions of the active fraction II were tested, each of which contains 98 to 100 per cent γ-globulin. Electrophoretic analyses had shown that fraction II-1 has a mobility of 1.42 and fraction II-3 a mobility of 1.88 in phosphate buffer at pH 7.7, ionic strength 0.2, protein concentration 2 per cent, and temperature +4 C.[3] Only one of these subfractions (II-3) showed selective activity, whereas the other one (II-1) failed to suppress nonsmooth types within concentrations that would correspond to normal blood levels. Table 3 illustrates the distribution of the same data when they are arranged on a weight basis.

It is noteworthy that neither of the "active" fractions per se shows selective activity in concentrations lower than 9 per cent, whereas whole plasma or serum suppresses dissociation when present in concentrations as low as 2 per cent. However, when the active fractions II, III-0, III-1, and III-2 were combined, it was possible to demonstrate dissociation-suppressing activity in concentra-

[3] These data, as well as those assembled in table 2, were supplied by Armour and Company.

tions close to 3 per cent, indicating that most, if not all, of the suppressive activity could be recovered in the active fractions indicated in tables 1 and 2.

It was ascertained that the inhibition of the establishment of nonsmooth variants in the presence of normal globulin fractions was not due to any shift in population dynamics; no significant deviations from the controls were found when the total number of cells was determined after various periods of growth in

TABLE 1

The effect of bovine plasma fractions on a "percentage of normal concentration" basis

AMOUNT OF FRACTION PER CULTURE EXPRESSED AS % OF ITS CONCENTRATION IN NORMAL PLASMA	DISSOCIATION INDICES AFTER ADDITION OF FRACTION									
	I	II	III-0	III-1	III-2	IV	IV-3,4	V	II-1	II-3
1660										
910										
332 - 380									7	
211 - 286	31	0						41	12	
100 - 182	31	0		34			48	63	19	
50 - 92	35	0	0	40	5		56	68	17	
20.9 - 40.0	66	1	0		5	28	40	76	10	
110 - 200	55		0	5	5	42	36	66	15	22
5.5 - 100	50	14		31	9	42	38	76	20	22
22 - 40	38	25	12	37	31	46	37	71	19	9
11 - 20	30	28	12	56	27	45	34	48	15	9
05 - 10	35	38	29	60	34	30		55	17	18
0.3 - 04		38	29	34	30	31		55	19	19
01 - 02	34	37						41	17	18
NONE	34	34	30	34	30	30	34	34	15	15
NUMBER OF TESTS	44	323	86	102	48	24	59	51	63	45

cultures containing the lowest effective amounts of fractions II or III-0 (table 3).[4] The dissociation-suppressing effect in the presence of normal bovine globulins can thus be interpreted only in terms of a truly selective action against the establishment of spontaneously arising nonsmooth variants.

This interpretation regarding the specific selective activity of certain globulins against the establishment of nonsmooth variants was further supported by the data obtained with mixed cultures. In agreement with the previously reported

[4] Whereas the lower effective concentrations of "active" globulin fractions did not affect population dynamics, higher concentrations of the same fractions, i.e., concentrations listed above the heavy line of table 1, produced slight bactericidal effects.

results with serum (Braun, 1946b), no increase in R types could be observed in the presence of active globulin fractions after 10 days of growth of populations that initially consisted of 95 per cent S and 5 per cent R types, whereas control cultures without globulin supplement showed a considerable increase in the percentage of rough over smooth during the same period.

When albumin and γ-globulin fractions obtained from human plasma were tested, it was again observed that the γ-globulin fraction, in concentrations corresponding to as little as 3 per cent plasma, would suppress dissociation, whereas the albumin fraction was again found to enhance the establishment of nonsmooth types.

TABLE 2

Composition of bovine plasma fractions

FRACTION	ELECTROPHORETIC ANALYSIS					ML PLASMA REPRESENTED BY 1 GM
	% ALBUMIN	% GAMMA-GLOBULIN	% BETA-GLOBULIN	% ALPHA-GLOBULIN	% FIBRINOGEN	
I		10	3		85	110
▨		98	2			180
▨	3		67	13	17	94
▨			4	96		400
‖II-2‖	5		75	20		500
IV	45		22	28		77
IV-3,4	9	6	38	47		360
V	97			3		70
II-1		100				1140
▨		98	2			265
▨	39	15	11	15	20	10

In addition, the human γ-globulin fraction here used showed a striking bactericidal effect upon *Brucella abortus*. The presence of the purified human γ-globulin, in amounts that would correspond to 7.5 per cent of plasma or more, proved to have a bacteriostatic effect in concentrations up to 30 per cent and a bactericidal effect in higher concentrations. This antibacterial effect was produced by non-filtered as well as Seitz-filtered preparations and was observed with virulent as well as avirulent smooth strains of *Brucella abortus*.[5]

It was found that the selective activity of either serum or its globulin fractions expresses itself only in buffered media. It was observed in buffered tryptose broth, beef extract broth, and broth containing both beef extract and tryptose (all buffered at pH 6.8). It was erratic or absent when the same media were used

[5] A similar effect has just been observed with equine γ-globulin fractions, whereas porcine and ovine fractions failed to produce antibacterial effects.

unbuffered. Since it is well known that unbuffered broth will become alkaline (approximately pH 8.6) after *Brucella abortus* has grown in it for several days, it is logical to assume that the active blood proteins are unable to exert their selective activity at higher pH ranges; the specificity of activity of proteins within narrow pH ranges is a well-established phenomenon.

Confirmation regarding the association of the selective factor with certain globulin fractions was obtained in experiments that indicated that serum con-

TABLE 3
The effect of bovine plasma fractions on weight basis

MILLIGRAMS OF FRACTION PER CULTURE	DISSOCIATION INDICES AFTER ADDITION OF FRACTION									
	I	II	III-0	III-1	III-2	IV	IV-3	V	II-1	II-3
100	31							63		
754-880										
500-560	31						41	68		
377-440										
189-280	35			34		28	48	76	7	
100-140	66			40		42	56	66	12	
50-75	55				5	42	40	76	19	
20-40	50	14	12	5	5	46	36	71	17	22
10-19	38	25	12	31	9	45	38	48	10	22
05-075	30	28	29	37	31	30	37	55	15	9
020-038	35	38	29	56	27	31	34	55	20	9
010-019	34	38		60	34			41	19	18
less than 008	34	37		34	30			42	16	19
NONE	34	34	30	34	30	30	34	34	15	15

taining anti-γ-globulin can inactivate the selective effect of γ-globulin. Dr. R. Cunha produced anti-γ-globulin by inoculation of bovine γ-globulin into rabbits. The addition of sufficient serum from these rabbits to cultures containing effective bovine γ-globulin concentrations resulted in the disappearance of the selective activity of the globulin fraction.

DISCUSSION

The ability of serum or plasma, or their above-described globulin fractions, to suppress the establishment of nonsmooth types attains special significance when the direct correlation between this *in vitro* activity and certain *in vivo* phenomena is observed. First of all, it is interesting to note that buffered media are necessary

to produce the selective effect *in vitro*, i.e., conditions are required which resemble the buffered environment existing *in vivo*. But more significant are the direct correlations between (1) the selective activity of serum factors *in vitro* and selective effects *in vivo* and (2) the absence of selective effects *in vivo* in certain species and the failure of serum from these species to suppress the establishment of non-S types *in vitro*. Equally suggestive is the temporary disappearance of the *in vitro* selective activity observed in serum samples that had been obtained from animals during certain periods after exposure to *Brucella*. These observations will be described in detail separately, but may be briefly illustrated here by the following examples: (1) When nonsmooth variants are inoculated into rabbits or guinea pigs, they cannot be recovered from the spleen several weeks later; animals inoculated with smooth clones, however, yield positive spleen cultures (Braun and Hauge, 1948). (2) When nonsmooth variants are inoculated into mice, they can be recovered from the spleen (Braun and Hauge, 1948). This *in vivo* selectivity in rabbits and guinea pigs is duplicated by the selective activity of serum from these species *in vitro*; the lack of *in vivo* selectivity of mice coincides with the absence of selective activity of mouse serum *in vitro*, i.e., non-S types are not suppressed when mouse serum is added to buffered broth cultures inoculated with a smooth clone.

It should be mentioned that additional data, collected during these studies, have substantiated previous observations that indicated that the selective effect is expressed only when the original bacterial population contains smooth types. That is, in a population consisting of 95 per cent S and 5 per cent R types, no increase in the percentage of R types takes place during 10 days of growth in cultures containing serum (or the active globulin fractions), whereas control cultures without serum will show a considerable increase in the percentage of rough over smooth during the same period (Braun, 1946b). The propagation of R, M, or other nonsmooth types, however, is not completely inhibited if serum or the active globulin fractions are added to pure R cultures, for example. Only if S types arise in these originally R cultures will the percentage of rough types decrease in the presence of the serum factor. The selective factor (or factors), therefore, merely creates an environment greatly favoring the S type without acting as a complete inhibitor for non-S types; nevertheless, its activity suffices to suppress completely the propagation of nonsmooth mutants, which arise during the growth of a smooth population, and it acts therefore as an efficient inhibitor of so-called dissociation, which occurs in most smooth clones in the absence of the globulin factor, e.g., in common laboratory media. From the foregoing, it may be concluded that buffered media containing normal serum or its appropriate globulin fractions represent an environment most nearly similar to normal *in vivo* conditions for *Brucella abortus*.

A few exploratory studies have indicated that the results described are not restricted to *Brucella abortus*. A limited number of experiments with *Staphylococcus aureus* (Hoerlein, 1948), *Pasteurella multicida*, and *Salmonella* suggest that the establishment of non-S variants of these species is similarly suppressed in the presence of normal serum or its γ-globulin fraction.

Investigations on the inactivation of the selective serum factor are now in progress. Considerable information on substances that are able to inactivate the selective activity *in vitro* is already available, and it is hoped that, if the selective factor suppressing nonsmooth (i.e., commonly avirulent) variants can be inactivated *in vivo*, this inactivation may possibly permit new means of disease control through the creation of an *in vivo* environment favoring the establishment of avirulent variants.

It has been mentioned above that decisive changes of selectivity are observed when sera from vaccinated or infected animals are used. For that reason the selective activity of *normal* serum or *normal* globulin fractions has been stressed. The manner in which this activity is modified, after exposure of susceptible hosts to *Brucella abortus*, will be detailed in the next paper of this series.

SUMMARY

A previously demonstrated heat-stable, filtrable factor that selectively suppressed the establishment of nonsmooth *Brucella abortus* variants in buffered broth cultures of smooth types, to which normal serum or plasma had been added, was found to be associated with the γ- and certain β-globulin fractions of normal bovine plasma.

The factor has also been observed in a γ-globulin fraction of human plasma, but, in contrast to the bovine fractions, the human γ-globulin fraction used in these studies simultaneously exhibited a strong antibacterial effect upon virulent and avirulent strains of *Brucella abortus*.

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STUDIES ON BACTERIAL VARIATION AND SELECTIVE ENVIRONMENTS

II. The Effects of Sera from Brucella-infected Animals and from Normal Animals of Different Species upon the Variation of Brucella abortus

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In previous reports (Braun, 1946, 1948) it has been shown that the addition of small amounts of normal serum, or certain of its globulin fractions, to buffered broth suppresses the establishment of nonsmooth variants (mutants) in growing smooth populations of *Brucella abortus*. The sera, or their fractions, which were used in these studies had been obtained from normal cows, guinea pigs, rabbits, or humans, i.e., from susceptible donors that had never been exposed to *Brucella*. However, when the serum was obtained from donors that had been infected with a virulent *Brucella abortus* culture, or had been vaccinated with strain 19 of *Brucella abortus*, results were obtained which differed from those previously presented for normal sera.

It was also found that normal sera from species with relative insusceptibility to *Brucella* infections failed to produce the selective effect previously described for normal sera from *Brucella*-susceptible species. These observations, with sera from infected animals and with normal sera from different species, will be reported in this paper.

EXPERIMENTAL DATA

As in previous studies on the *in vitro* effect of sera, varying amounts of Seitz-filtered serum were added to 5 ml of buffered beef extract broth cultures, usually in concentrations from 2 to 20 per cent; the most significant data were obtained in cultures containing 5 to 10 per cent serum. In the experiments with sera from infected animals, these cultures were inoculated with either a smooth clone isolated from strain 19, yielding approximately 30 per cent nonsmooth types after 10 days of growth in cultures without serum (dissociation index = 30 per cent) or a virulent S culture with a dissociation index of 22 per cent. In the experiments with sera from different species, only the smooth clone isolated from strain 19 was used. All data are based on at least triplicate tests with each concentration of the various serum samples.

The effect of sera from Brucella-infected animals. Sera from 42 cows were tested. Nine of these cows had never been exposed to a virulent strain of *Brucella*

abortus and had not been vaccinated with strain 19 (a strain of low virulence which is commonly used for vaccination). The addition of serum from any of these normal animals to buffered broth cultures suppressed the establishment of nonsmooth variants during the 10-day growth period of originally smooth populations, both in cultures started with 19S or virulent S (see examples in table 1). However, the addition of serum from any of 20 animals that had been vaccinated

TABLE 1

Examples of the effect of sera from normal, infected, and vaccinated cows upon the establishment of variant types in vitro

STATUS OF COW	AMOUNT OF SERUM ADDED TO 5 ML OF BROTH	DISSOCIATION INDEX (%) OF CULTURES INOCULATED WITH		REMARKS
		S 2583 (From strain 19)	S 6232 (Virulent)	
Normal......	0.5	0	0	Nonvaccinated and noninfected
Normal.......	0.5	0	0	Nonvaccinated and noninfected
Vaccinated ...	0.5	42 R* & M*	98 I*	Vaccinated 17 months before testing
Vaccinated .	0.5	24 R* & M*	70 I*	Vaccinated 24 months before testing
Vaccinated....	0.5	0	0	Vaccinated 37 months before testing
Infected.. .	0.5	0	25 R & M	First positive culture obtained 15 months before testing
Infected.. .	0.5	0	15 R & M	First positive culture obtained 19 months before testing
Infected and vaccinated...	0.5	21 R & M	36 I, R, & M	Positive culture 12 months, vaccinated 11 months before testing
Infected and vaccinated...	0.5	26 R & M	39 I, R, & M	Positive culture 17 months, last vaccinated 12 months before testing
Controls .	None	32 R & M	22 R & M	

* Predominant types of variants found.

within 28 months prior to bleeding failed to suppress the establishment of nonsmooth types (table 1). In cultures originally inoculated with 19S, varying percentages of rough and mucoid types were observed after 10 days of growth; in cultures inoculated with the virulent S a high percentage of intermediate types were found. (These intermediate types proved to be avirulent for guinea pigs.) Such results were obtained both with sera containing detectable agglutinins and with sera that had ceased to produce agglutination reactions. Serum obtained from six animals that had been vaccinated 37 months or more prior to

bleeding behaved like normal serum, i.e., suppressed the establishment of non-smooth types. Serum obtained from six animals that had been naturally infected but had never been vaccinated[2] produced different results with the two test cultures; when added to cultures of virulent S these sera failed to suppress the establishment of nonsmooth variants, but when added to cultures of 19S no nonsmooth types established themselves, i.e., "infected" serum had the same effect as "normal" serum upon organisms of strain 19 (table 1). Serum samples obtained from seven animals that had been vaccinated with strain 19 and had also been naturally infected failed to suppress the establishment of nonsmooth variants in originally virulent S or avirulent (strain 19) S cultures; however, the types found in virulent cultures after the addition of "infected plus vaccinated" serum contained a large number of intermediate types in addition to R and M, whereas R and M types only were observed after the addition of "infected" serum (table 1).

These effects produced by sera from exposed cows were confirmed with a limited number of human sera, including samples from persons having had contact with strain 19 or virulent cultures (but showing no clinical signs of brucellosis), and with a number of sera from infected guinea pigs and rabbits. The results were identical with those from the examples compiled in table 1, and the general effect of serum from normal donors and donors exposed to strain 19, virulent cultures, or a combination thereof, upon the variation of *Brucella* can now be summarized as shown in table 2. The potential value of these results for diagnostic procedures is evident, especially since the strikingly different effects produced by sera from infected and vaccinated donors is independent of the presence of agglutination titers, as witnessed by the results obtained with sera from vaccinated animals that had ceased to show detectable agglutinins.

The lack of any correlation between the presence of agglutinins and the change in selective serum factors after exposure has been substantiated by observations on serum samples obtained periodically after inoculation of strain 19S into cows and rabbits. It was found that sera from these inoculated animals maintain their selective effect until approximately 4 weeks after the appearance of agglutinins, i.e., sera obtained during the early postexposure period suppress the establishment of nonsmooth types in originally smooth broth cultures exactly as has been described for sera from normal, nonexposed animals. Serum obtained more than 4 weeks after the appearance of agglutinins, however, will permit the establishment of nonsmooth types, as shown in tables 1 and 2; and, as already mentioned, this altered selective effect can be observed for at least 2 years after the disappearance of agglutinins.

It should be added that the percentage of nonsmooth types which establish themselves in cultures to which "exposed" serum has been added can actually be higher than the percentage of nonsmooth types found in control cultures without serum inoculated with the same S clone. This has been observed especially

[2] Blood cultures from these animals had been positive approximately 10 months prior to bleeding; no attempts were made to culture blood samples at the time blood was obtained for the studies here reported.

when serum obtained from animals within the early months after exposure was used. However, the data are insufficient to determine whether the percentage of nonsmooths establishing themselves within "exposed serum broth cultures" may serve as an indicator of the period that has elapsed since exposure. In any

TABLE 2

Summary of the effects of sera from donors exposed in different ways

	EFFECT OF ADDITION OF SERUM UPON THE ESTABLISHMENT OF VARIANT TYPES IN BROTH CULTURES OF	
SERUM DONOR'S STATUS	S 2583 (from 19)	S 6232 (virulent)
Normal...	—*	—
Vaccinated (exposure to strain 19S) ..	+† R & M types	+ I types
Infected (exposure to virulent S).....	—	+ R & M types
Infected and vaccinated.....	+ R & M types	+ I, R, & M types

* — = no nonsmooth types after 10 days of growth.
† + = nonsmooth types present after 10 days of growth.

TABLE 3

The effect of the addition of sera from various species upon the establishment of nonsmooth types ("dissociation") in broth cultures inoculated with a smooth clone

NORMAL SERA FROM THE FOLLOWING SPECIES	
Suppress dissociation *in vitro*	Fail to suppress dissociation *in vitro*
Man	Hamster
Cow	Rat
Rabbit	Mouse
Guinea pig	Chicken
Hog	Quail
Cat	Duck
	Pheasant

Example

TYPE OF SERUM ADDED	AMOUNT	D.I.(%)
Normal human.	4%	0
Normal bovine.......	4%	0
Normal rat.	4%	22
None.............	—	22

event, these last-mentioned observations indicate that not only is there a post-exposure disappearance of the selective serum effect, but that during certain periods after exposure, at least, the establishment of nonsmooth types may be enhanced.

The effect of sera from different species. Table 3 summarizes the results obtained from over 300 cultures. It appears unnecessary to go into detailed descriptions

of the individual observations. It can be seen that normal sera from 6 of the 13 species tested suppressed the establishment of nonsmooth types, whereas sera from the other 7 species failed to do so, i.e., in the presence of the latter sera the establishment of nonsmooth types proceeded exactly as in control cultures without serum.[3] It is obvious that there is a remarkable agreement between the known susceptibility to *Brucella* infections of all the species from which "dissociation-suppressing" serum is obtained and the known relative insusceptibility to *Brucella* infections of those species that lack the selective serum factor.

<div align="center">DISCUSSION</div>

These results demonstrate that in *Brucella*-susceptible species the selective serum factor, suppressing the establishment of nonsmooth types *in vitro*, is temporarily inactive after infection, and that in species with relative insusceptibility to *Brucella* infections the selective serum factor is entirely lacking. This means that after infection or vaccination the serum activity of susceptible species becomes temporarily like that of nonsusceptible species.

If these observations *in vitro* reflected similar effects *in vivo*, one would be tempted to suggest a possible causal relationship between susceptibility and serum selectivity. Actually, it has been possible to demonstrate that the difference in selectivity *in vitro* of normal sera from different species is directly correlated with differences of selective effects *in vivo* of the same species (Braun and Hauge, 1948): nonsmooth types inoculated into mice (serum nonselective *in vitro*) can be recovered from the spleen after several weeks whereas the same nonsmooth types cannot be recovered after inoculation into guinea pigs or rabbits (whose normal serum suppresses the establishment of nonsmooth types *in vitro*). These observations thus support the suggestion that susceptibility is correlated with the presence of a normal serum factor suppressing the establishment of non-smooth, usually avirulent types, whereas a lack of this factor will be associated with increased resistance.

One might speculate that after exposure to *Brucella* the establishment of the virulent smooth type would be initially forced in all those species listed on the left side of table 3 (susceptible species), whereas after the infection of, for example, a mouse or duck with any type (S, R, or M, etc.) the establishment of the avirulent, nonsmooth types would be favored in the absence of any selective factor. Furthermore, the disappearance of the selective suppression *in vitro* when serum from infected, susceptible animals is used suggests that after exposure *in vivo* selectivity may similarly change and permit the establishment of avirulent, nonsmooth variants. This would coincide with the known increased resistance to subsequent infections of infected or vaccinated animals and is supported by the reports on the isolation of variant types of various pathogens from carriers or convalescent hosts.

An additional instance of change in selective serum activity, presumably associated with changes in resistance, was observed during late pregnancy in a cow.

[3] In cultures containing 10 to 20 per cent of mouse serum the establishment of nonsmooth types was actually enhanced.

Serum from this animal, which had served as donor for normal serum for over a year, suddenly failed to suppress dissociation *in vitro*. This change occurred during the sixth month of pregnancy and was found to persist until calving, after which the former selective activity returned immediately. During a subsequent pregnancy of the same animal the same type of change in serum activity after the sixth month of gestation was observed. The clinical literature contains many references to increased resistance to various infections during late pregnancy (Perla and Marmorston, 1941), and it becomes a challenging question whether this altered resistance could be associated with changes in selective serum activity as observed in this one animal.

The mechanism responsible for this temporary loss of selective *in vitro* activity of serum from susceptible animals after infection is still obscure. After some early and preliminary observations on the effect of sera from infected animals (Braun, 1946), it was suggested that the suppression of S types due to the presence of S antibodies may be stronger than the suppression of rough and mucoid types by factors normally present in serum, and so-called dissociation may thereafter occur in the presence of antiserum. However, the more recent observations on the lack of correlation between the presence of agglutinins and alteration of the serum effect make such an explanation unlikely. Some other mechanism must be looked for. In this connection it may be of interest to mention some preliminary observations on absorption experiments which indicate the complexity of changes occurring after infection. It was observed that the selective activity of normal serum from *Brucella*-susceptible species cannot be absorbed by exposure of the serum to R and M types, the types that are suppressed by normal sera. However, if serum is obtained from such animals during that brief period after infection when the serum still retains its selective effect in the presence of agglutinins, the non-S-suppressing activity can be absorbed by exposure to R and M for 3 hours at 37 C.

In conclusion it may be pointed out that the different results obtained when sera from *Brucella*-susceptible animals exposed to strain 19S or a virulent S were used in cultures inoculated with a 19S clone or a virulent S clone not only provide an effective tool for differential diagnosis, but appear to indicate a lack of close relationship between strain 19 of *Brucella abortus* and a virulent culture of the same species.

SUMMARY

The previously. described selective activity of normal sera from *Brucella*-susceptible species suppressing the establishment of nonsmooth *Brucella abortus* types in smooth broth cultures was found to be lacking in sera from infected animals of these susceptible species, and was also found to be absent in sera from normal animals of nonsusceptible species.

The change in activity of sera from infected animals belonging to susceptible species occurred approximately 4 weeks after agglutinins were first detected and persisted for at least 2 years after their disappearance.

Different effects were produced by sera from infected or vaccinated animals

upon the variation of a virulent culture or a strain with low virulence (strain 19), respectively. This suggests that the effect of an animal's serum upon *in vitro* variation may be utilized for diagnostic tests, indicating the status of an animal in regard to *Brucella* infection or past vaccination independently of agglutination titers.

The possibility of a causal relationship between serum selectivity and susceptibility has been discussed.

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VARIATIONS IN NORMAL AND BRUCELLA-IMMUNE RABBIT SERUM AS DETERMINED BY PAPER-PARTITION CHROMATOGRAPHY

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Chromatography has been divided into five basic methods, of which the paper-partition type is the only one of importance here. This method was developed as a result of previous work with partition chromatography by Consden *et al.* (1944; Consden, 1948) in which silica gel was used as a supporting medium. It was modified, with cellulose in the form of filter paper as a stationary support. In this method, filter paper strips carrying the sample to be assayed were hung from a trough containing the solvent, the whole system being in an atmosphere saturated with respect to solvent and water vapors. The solvent siphoned down the strips, and after a suitable length of time the amino acids were revealed as color bands by being dried, sprayed with a ninhydrin solution, and dried again. The techniques of partition chromatography were modified by Horne and Pollard (1948) in their experimental work on the identification of streptomycin. They employed an ascending technique. The procedure was basically the same as the one just described except that the container of solvent was placed at the bottom of the filter paper strips, thus causing the solvent to climb by capillary ascent. The latter method is advantageous with respect to simplicity of apparatus, constancy of results, and, particularly, the ease with which a large number of analyses may be run.

EXPERIMENTAL METHODS

The problem at hand was to determine qualitatively (within the limits of the method) the amino acid content of normal rabbit serum and rabbit anti-*Brucella* serum, employing the ascending technique of chromatography. Rabbits maintained under standard dietary conditions were subjected to a series of injections so that, upon termination, the concentration of agglutinins in the blood had reached a titer of 1:1,280. The antigen employed was prepared, using standard procedures, by growing cultures of *Brucella abortus*, strain B.A.I. 19, upon Difco tryptose agar. The growth was removed by being washed with a physiological saline solution. The cell suspension was heat-killed and standardized to a no. 6 McFarland nephelometer tube.

Prior to immunization the animals were bled, and on the seventh day after the cessation of injections they were bled again. In both instances a clot was allowed to form and the serum was separated by centrifugation. Both normal and immune sera were hydrolyzed by mixing 1 ml of the given serum and an equal amount of 12 N hydrochloric acid and autoclaving at 121 C for 2 hours. Immediately there-

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after the samples were evaporated to dryness by being placed in a desiccator with a drying agent; aspiration was employed for a suitable length of time. The residues were allowed to remain under partial vacuum overnight and were brought back to the original total volume by the addition of triple-distilled water. After the water and residues were thoroughly mixed, the suspensions were neutralized with silver oxide; Congo red paper was used as an indicator. The hydrolyzates

TABLE 1

Rf values for various amino acids in aqueous solutions as established at the University of Tennessee

AMINO ACIDS	REDISTILLED PHENOL SOLVENT	COLLIDINE AND LUTIDINE SOLVENT
Glycine	0.43–0.44	0.22–0.23
Alanine	0.58	0.27–0.29
Valine	0.74–0.75	0.46–0.53
Isoleucine	0.82	0.52
Leucine	0.83–0.87	0.58–0.61
Phenylalanine	0.90–0.92	0.65–0.66
Tyrosine	0.66–0.68	0.79–0.84
Serine	0.35–0.38	0.29–0.32
Threonine	0.48	0.31–0.32
Hydroxyproline	0.69	0.42
Proline	0.84–0.85	0.29–0.30
Tryptophan	0.84	0.73–0.77
Histidine	0.72–0.76	0.25–0.26
Arginine	0.88	0.10–0.12
Lysine	0.80	0.07–0.08
Aspartic acid	0.16	0.13–0.15
Glutamic acid	0.27–0.31	0.17–0.19
Cystine	0.15–0.18	
Cysteic acid	0.10	0.35–0.40
Methionine	0.81–0.82	0.53–0.56
β-Alanine	0.64	0.18
Asparagine	0.42–0.43	0.17–0.18
Histamine·HCl	0.87	0.46–0.51
Taurine	0.41	0.40
α-Amino-isobutyric acid	0.65	0.42
Histamine base	0.80	0.53

$$Rf \text{ value} = \frac{\text{Movement of color band}}{\text{Total movement of solvent}}.$$

were filtered and the filtrates applied directly to the chromatographic strips

Two hundredth-ml portions of the hydrolyzates were applied to Whatman no. 1 filter paper strips cut to dimensions of approximately 3.8 cm by 45.7 cm. The solvents used were 85 per cent phenol in an ammonia atmosphere and 35 per cent collidine and 35 per cent lutidine in a water atmosphere. The solvent fronts were allowed to ascend for 24 hours in each case, and the strips were dried and developed with 0.1 per cent ninhydrin in 83 per cent butanol. The Rf values were computed and compared with Rf values of known amino acids determined in the laboratory for the purpose of identification (table 1).

RESULTS

Tentative identification of the amino acids in the normal and immune sera indicated a pronounced variation; however, it is well known that a masking of color bands by closely related acids will result in invalid interpretation of Rf values. In addition, a fluctuation of temperature and humidity tends to affect the accuracy when the bands lie very close to each other. Therefore, the differences in the two sera, demonstrated when phenol was used as a solvent, and in those color bands with Rf values of 0 to 0.73 when collidine-lutidine was employed as a solvent were not considered to be of major importance. In the case of the strips containing the immune serum which were run, using the collidine-
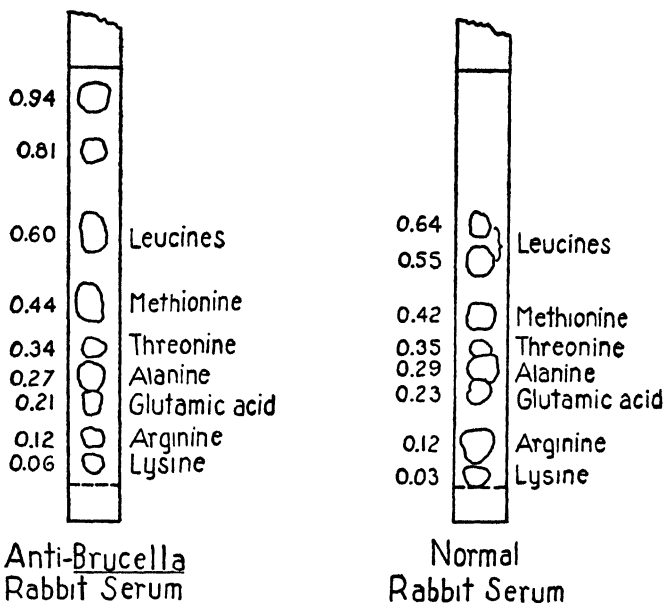
Figure 1. Chromatographic results when collidine-lutidine was employed as a solvent.

lutidine system, there appeared two color bands with Rf values of 0.81 and 0.94. These values were higher than any reported for this solvent. The distance by which these bands were separated from others on the strips almost eliminated the possibility of confusing them because of masking or environmental conditions. Strips containing samples of the normal serum failed to show corresponding color bands, even when run simultaneously with the same solvent and in the same piece of apparatus (figure 1).

Several attempts have been made to identify the two color bands with high Rf values. Their location indicated that the phenolic group was involved and Millon's test showed its presence in reactive quantities. The normal sera produced a negative reaction when tested with Millon's reagent. This was considered as indicative, if not conclusive evidence, of a chemical change in the two types of sera. Although tyrosine, which bears a phenolic ring, failed to appear in either the normal or immune rabbit seria, it was recognized that this amino acid has

a number of breakdown products. With this in mind, tyrosinase, a copper-bearing enzyme, was mixed with tyrosine in a water solution and incubated for 120 hours at 37 C; chromatographic strips were run with the resulting products, with collidine-lutidine as a solvent. An Rf value characteristic of tyrosine was all that was indicated. It was realized that this short period of incubation was not sufficient to give conclusive evidence that would eliminate the possibility of a tyrosine breakdown product being involved as one of the unknown compounds. Tyramine hydrochloride in a water solution was also employed. When used alone, it gave an Rf value of 0.97, but when mixed with normal serum the Rf value dropped to 0.95 or close to that of the topmost band on the chromatogram of the immune serum. Whether it can be established that tyramine exists in rabbit anti-*Brucella* sera remains to be proved.

When sections designated as in table 2 were cut from undeveloped chromatograms of normal and immune sera and eluted with 0.5 ml of metal-free distilled water, some of these gave positive reactions with the sodium diethyl-dithio-

TABLE 2

Results of application of sodium diethyl-dithiocarbonate to elutions of sections of undeveloped paper strips as a test for copper or iron

	SECTION OF PAPER STRIP ABOVE SOLVENT FRONT	SECTION BETWEEN RF 0.92-1.0	SECTION BETWEEN RF 0.78-0.85	SECTION BETWEEN RF 0.30-0.40
Normal rabbit serum....	−	+	−	−
Rabbit anti-*Brucella* serum..... ..	−	+	+	−

carbonate test (Waterhouse, 1945), signifying the presence of copper or iron (table 2). This test is sensitive to copper in concentrations of 1 part per 100,000,000, a sensitivity not approached in the case of iron. No attempt has been made as yet to evaluate the significance of these findings.

The agglutinin adsorption technique was applied to the immune serum, and after centrifugation of the cells the supernatant serum was subjected to acid hydrolysis and neutralization. The sample was run on paper strips according to the procedure previously described. The color band with an Rf value of 0.81 was no longer present.

Although the work to date has been primarily exploratory, it may be concluded that there is a difference in normal rabbit sera and rabbit anti-*Brucella* sera which is chromatographically demonstrable, and· that chromatography gives evidence of being an exceedingly useful tool in providing new information relative to the composition of antibodies.

SUMMARY

Paper-partition chromatography was employed for the determination of the amino acid content of normal rabbit serum and *Brucella*-immune rabbit serum. The normal rabbits were bled prior to immunization with *Brucella abortus*

and again after the agglutinin titer reached 1:1,280. Both normal and immune sera were hydrolyzed with 12 N hydrochloric acid, and 0.02 ml of the hydrolyzate were placed on paper strips for chromatographic analysis. Two different solvent systems were employed—(1) distilled phenol and (2) collidine and lutidine. The amino acids of normal serum were identifiable in the *Brucella*-immune serum, but the latter also showed the presence of two additional amino acids or compounds containing groups specific for the ninhydrin developer when the collidine-lutidine solvent was used.

With the Millon test it was shown that the phenolic group was present in the immune serum and absent in the normal serum. When the sodium diethyl-dithiocarbonate reagent was used to test elutions of given portions of the paper strips, copper or iron was indicated in some of the areas and absent in others. The color band with an Rf value of 0.81 was absent after adsorption of the immune serum with *Brucella abortus*.

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NITROGEN FIXATION BY PHOTOSYNTHETIC BACTERIA[1]

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The results from a series of studies concerned with the influence of molecular hydrogen on biological nitrogen fixation (Wilson, 1940) led to the suggestion that hydrogenase, the enzyme activating H_2, was associated in some way with the nitrogen-fixing enzyme system. Although obscure in the symbiotic combination of leguminous plant and *Rhizobium* (Wilson, Burris, and Coffee, 1943), the association was so noteworthy with *Azotobacter* that it suggested that any organism possessing hydrogenase might be a potential nitrogen fixer. At that time we tested three organisms using N^{15} as a tracer: *Escherichia coli; Proteus vulgaris*, Hoberman's (1942) strain that catalyzes the hydrogen-exchange reaction; and *Scenedesmus*, Gaffron's (1944) strain that, with appropriate treatment, switches from photosynthesis to photoreduction. This limited survey revealed no new nitrogen-fixing agents, but recently Gest and Kamen (1949*a,b*) and Kamen and Gest (1949) have provided a most interesting example in *Rhodospirillum rubrum*, a nonsulfur purple bacterium. Because of the significance of their findings, we undertook to check their more important observations. On their invitation[2] we first made a joint experiment testing the ability of a heavy suspension of the organism to fix N_2^{15}. The results were so clear-cut that it appeared likely that more conventional procedures using growing cultures on a low-nitrogen medium with a light inoculum and analysis by the Kjeldahl method would readily detect fixation by this organism. Independent tests in our three laboratories have confirmed this prediction.

The results from typical experiments are summarized in table 1. The medium consisted of KH_2PO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; DL-malic acid, 3.5 g; Difco yeast extract, 0.2 g; biotin, 20 μg; trace metals (plus Mo) according to Hutner (1946); distilled water to 1 liter after the pH was brought to 7 with 6 N KOH. The inoculum was 2 per cent by volume of a culture grown on a similar medium (0.8 g sodium citrate added and the quantity of yeast extract doubled). Incubation was from 3 to 6 days at 25 C. Light was from a 200-watt mazda bulb about 30 inches from the cultures. Accompanying growth of the organism was an increase in alkalinity, but the pH seldom exceeded 8.0 at harvest. Little or no growth was noted anaerobically in the dark; aerobically, both in dark and light, growth was definite but apparently restricted to the combined nitrogen

of the medium. Fixation thus apparently accompanies photoreduction (anaerobic, light); at least heterotrophic fixation (aerobic, dark) was insufficient to be detected with the Kjeldahl method. With N_2^{15}, evidence of a small though significant fixation has been observed; this point will require further testing.

TABLE 1

Nitrogen fixation and photoreduction by Rhodospirillum rubrum

CONDITIONS OF INCUBATION	EXPERIMENT I	EXPERIMENT II	EXPERIMENT III
Time in days....	4	5	4
Anaerobic, light	77*	88†	77
Anaerobic, dark......	22	22	20
Aerobic, light........	22	25	18
Aerobic, dark......	22	21	17
Uninoculated control......	20	21	20

In experiment III, 0.05 M lactate were used instead of malate.

* All data are final total N in micrograms per ml.

† 101 micrograms per ml after 6 days.
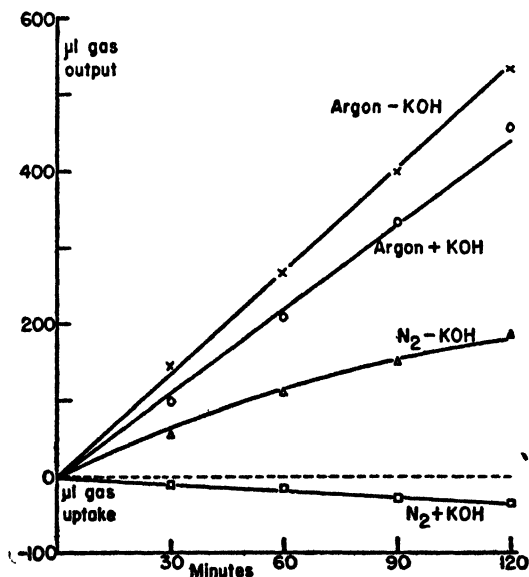
Figure 1. Suppression by molecular nitrogen of hydrogen evolution from *Rhodospirillum rubrum*. Forty-eight-hour culture of organism washed and suspended in pH 6.6 M/20 phosphate buffer; 2 ml of this suspension (1.24 mg cell N) plus 5 mg neutralized malic acid in 0.2 ml solution and 0.15 ml 20 per cent KOH (when used) placed in each Warburg flask. Flasks flushed with argon or nitrogen and gas exchange measured at 30 C under mazda lamps at light intensity of approximately 100 foot-candles.

One of the most significant observations made by Kamen and Gest was the inhibition of hydrogen evolution in *Rhodospirillum* by molecular nitrogen—the counterpart of hydrogen inhibition of nitrogen fixation. This critical observation likewise has been confirmed, as shown by the data in figure 1. In the presence of

N_2 the hydrogen evolution observed under argon was entirely suppressed. The gas uptake observed in an N_2 atmosphere in the presence of KOH probably arises from nitrogen fixation; during the 2-hour period such an uptake would constitute a 3.5 per cent increase in the cellular nitrogen present. The gas evolved from the growing culture before it was harvested for the manometric test formed an explosive mixture with O_2. The washed cells had a Q_{H_2} (N) of 185.

The close agreement of our experiments with those reported by Kamen and Gest is in itself noteworthy, since ability to duplicate results has scarcely been characteristic of the research on biological nitrogen fixation. Of greater importance is that the findings emphasize again that organisms known to contain hydrogenase, e.g., *Thiorhodaceae*, *Vibrio desulfuricans*, *Acetobacter peroxydans*, and certain strains of the luminous bacteria should be surveyed for the possible occurrence of hitherto undetected nitrogen fixers.

SUMMARY

The observations of Gest and Kamen that *Rhodospirillum rubrum* fixes molecular nitrogen and that the fixation is associated with photoreduction and the hydrogen metabolism of the organism have been confirmed. The results suggest that other organisms possessing hydrogenase should be examined for nitrogen fixation.

ADDENDUM TO PROOF

Of the untested hydrogenase-containing organisms, representatives of the *Thiorhodaceae*, because of their close relationship to *Rhodospirillum*, appear to be the most likely ones able to fix N_2. Professor C. B. van Niel kindly furnished us with strains of two genera to test this crucial point: *Chromatium*, a purple sulfur bacterium; and *Chlorobacterium*, a green sulfur bacterium. While this manuscript was in press results from Kjeldahl trials provided definite evidence that nitrogen fixation among the photosynthetic bacteria is not limited to the *Athiorhodaceae*. The results from a typical experiment were:

Chromatium: refrigerated controls, 12 μg/ml
in H_2—15 (4 days); 12 (5 days)
in N_2—32 (4 days); 43 (5 days)
Chlorobacterium: refrigerated controls, 12
in H_2—12 (4 days); 14 (5 days)
in N_2—30 (4 days); 32 (5 days)

Experiments with isotopic N_2^{15} are now in progress.

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THE SYNTHESIS OF NUCLEIC ACIDS IN CULTURES OF ESCHERICHIA COLI, STRAINS B AND B/R

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Although many investigations concerning the content of nucleic acids in bacterial cells have been reported (Stacey, 1948; Belozersky, 1947; Boivin, 1948), no systematic study of nucleic acid synthesis during growth was undertaken until the studies of Malmgren and his co-workers (1945, 1947a,b,c,d,e) and of Cohen (1948).

The results of the study of Malmgren and co-workers showed that the total nucleotide content of the individual cells of a culture changed during the growth of the culture, being at a maximum in cells near the end of the lag phase of the culture cycle. Cohen observed the rapid synthesis of ribose nucleic acid in his cultures following inoculation. These observations are in agreement with those of Belozersky and Boivin, who observed that cells from young cultures contained more nucleic acid than those from old cultures.

Preliminary to a study of the effects of ultraviolet and X radiation on the metabolism of nucleic acids in *Escherichia coli*, strains B and B/r, the present study was undertaken to establish, by means of microchemical procedures and with the use of radiophosphorus, the normal patterns of synthesis of ribonucleic and desoxyribonucleic acids in cultures of these microorganisms.

MATERIALS AND METHODS

Cultures and media. The B strain of *Escherichia coli* employed was obtained from Dr. E. H. Anderson, of this laboratory, and the ultraviolet-resistant B/r strain (Witkin, 1946, 1947) was obtained from Dr. Seymour Benzer, also of this laboratory. Both of these cultures were maintained at refrigerator temperature in stock culture on nutrient agar slants. The stock cultures were transferred at monthly intervals.

All cultivations except those in the experiments proper were accomplished in Difco nutrient broth plus 0.5 per cent NaCl or on salt broth plus 1.5 per cent Difco agar. All platings for bacterial count were made by making appropriate dilutions in nutrient broth, adding a portion of the highest dilution to 3.3 ml of melted (48 C) 0.6 per cent nutrient agar, and pouring the molten agar on the surface of a fresh nutrient agar plate. Platings were in duplicate, and colony counts were made after 20 hours' incubation at 37 C.

In some experiments a synthetic medium of the following composition was employed:

L-Glutamic acid (Eastman)	2.0 g
Glycine (Eastman)	7.5 "
Glucose	2.0 "
Glycerol	1.0 "
NaCl	5.9 "
MgSO₄	0.1 "
CaCl₂	0.1 "
Distilled water	1,000 ml

Trace elements were added to give the following concentrations in milligrams per liter of medium: boron, 0.01; copper, 0.1; iron, 0.2; manganese, 0.02; molybdenum, 0.02; and zinc, 2.0. The pH was adjusted to 7.4 with normal NaOH, and the medium was sterilized in 500-ml volumes at 15 pounds pressure for 15 minutes. Just prior to use, 1.0 ml of sterile 1.0 per cent KH_2PO_4 was added per 100 ml of medium.

Chemical analysis. Samples containing from 5.0×10^9 to 1.0×10^{10} cells were removed from the experimental cultures and added to 40-ml heavy-walled centrifuge tubes containing enough cold 50 per cent trichloroacetic (TCA) to give a final concentration of 10 per cent. The tubes were then centrifuged (usually within 15 minutes of sampling) at 2,500 rpm for 20 minutes, the supernatant was discarded, and the sedimented cells were allowed to drain.

The sedimented cells were hydrolyzed with 2.0 ml of 5 per cent TCA in a boiling water bath for 30 minutes, made up to a volume of 2.0 ml again with 5 per cent, and recentrifuged at 2,500 rpm for 15 minutes. The supernatant was poured off into another tube, and portions of it were analyzed for ribose by the orcinol test (Kerr and Seraidarian, 1945) and for desoxyribose by the diphenylamine test (Sevag *et al.*, 1940).

The procedure for the orcinol test was as follows: to 2.0 ml of a suitable dilution of the TCA extract in a colorimeter tube were added 5.0 ml of 0.02 per cent $FeCl_3$ (in conc. HCl) and 0.3 ml of 10 per cent orcinol (in 95 per cent EtOH). After the tube contents were mixed, the tube was heated for 20 minutes in a boiling water bath, cooled, and read in an Evelyn colorimeter using a 660 filter. The standard used for these estimations was a sample of Schwarz Laboratories yeast nucleic acid.

The estimations of desoxypentose were made as follows: to 1 ml of TCA extract in a small tube were added 2.5 ml of the diphenylamine reagent (1.0 g of Eimer and Amend diphenylamine dissolved in 2.0 ml conc. H_2SO_4 and 98 ml redistilled glacial acetic acid), and the tube was placed in a boiling water bath for 5 minutes. It was cooled and read in a Beckman spectrophotometer at 5,400 A. The standard used in these estimations was a sample of desoxyribose nucleic acid prepared according to Bang-Hammarsten procedure.

In each experiment a volume of medium, usually 200 ml, was prewarmed to 37 C in a water bath and seeded with 17 ml of a 20-ml, 24-hour nutrient broth culture. This yielded an initial bacterial count between 1.0×10^8 and 4.0×10^8

per ml of seeded medium. A sample was removed for bacterial count and chemical analysis, and air was then bubbled through the culture. At intervals thereafter samples were removed for bacterial count and chemical analysis without interruption of the air flow.

Experiments were performed in which the medium seeded from the 24-hour broth culture was the synthetic medium described above and 0.5 per cent NaCl nutrient broth.

In addition, two experiments were performed using the radioactive isotope of phosphorus, P^{32}, as an indicator of nucleic acid synthesis. In these experiments the usual procedure was followed using the synthetic medium. However, radiophosphorus (0.3 microcurie per ml of medium) was used in place of the phosphate usually added. The samples taken from the culture during growth were fractionated according to a modification of the Schmidt-Tannhauser procedure (1945), and the fractions, after digestion with H_2SO_4 and H_2O_2, were examined for isotope concentration. The samples were precipitated with 5 per cent TCA and the precipitate washed three times with 2.0-ml volumes of 5 per cent TCA. The initial supernatant and the washings were combined to form fraction A. One ml of distilled water was added to the precipitate remaining after fraction A, and it was then extracted three times with 4.0-ml volumes of 95 per cent ethanol and the extracts were combined to form fraction B. The precipitate remaining after alcohol extraction was solubilized and hydrolyzed in 2.0 ml of 1 N KOH at 37 C for 2 hours and the solubilized material then reprecipitated with 0.4 ml of 6 N HCl and centrifuged. The supernatant and three 2.0-ml 5 per cent TCA washings were combined to form fraction C. It has been found that a 2-hour period of solubilization and hydrolysis is sufficient to separate RNA from DNA and that no inorganic phosphate is formed in this period. RNA is not reprecipitated with 0.4 ml 6 N HCl. The precipitated material remaining after fraction C was hydrolyzed in 5.0 ml of 5 per cent TCA at 90 C for 30 minutes. The supernatant after centrifugation and three 2.0-ml 5 per cent TCA washings were combined to form fraction D. The precipitate remaining after fraction D was dissolved in concentrated H_2SO_4 and formed fraction E.

All fractions were digested with H_2SO_4 and H_2O_2, and after appropriate dilution, dried in capsules and the P^{32} content estimated with a conventional Geiger-Müller counter.

RESULTS

Representative results are shown graphically in figures 1, 2, 3, and 4. Figure 1 illustrates the synthesis of ribose nucleic and desoxyribose nucleic acids in cultures of *E. coli*, strain B, upon transfer from broth to the synthetic medium, and figure 2 the synthesis upon transfer to fresh broth. Figures 3 and 4 illustrate the change in average nucleic acid content of the cells during these experiments. Figure 5 illustrates the synthesis of nucleic acids by cultures of *E. coli*, strain B/r, upon transfer to synthetic medium. Figure 6 illustrates the change in average nucleic acid content of the B/r cells during growth.

From these results it appears that considerable amounts of nucleic acid are

synthesized during the lag period, before actual cell multiplication has commenced. When the data are considered from the aspect of the cell, it is observed
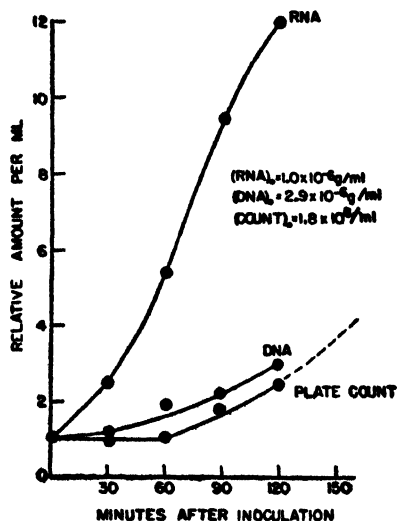
Figure 1. Synthesis of nucleic acid by *E. coli*, strain B, by cells transferred to synthetic medium.
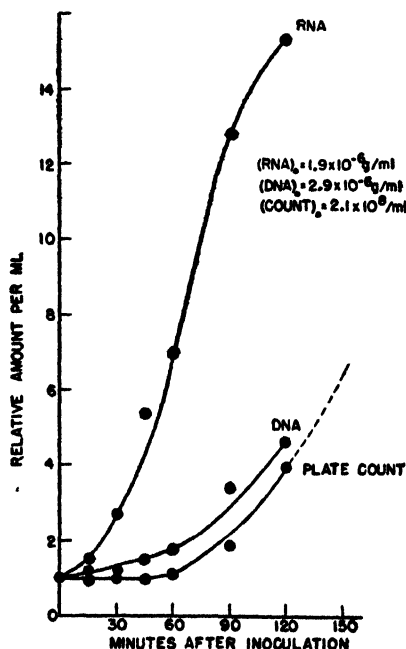
Figure 2. Synthesis of nucleic acid by *E. coli*, strain B, by cells transferred to fresh broth.

that the amount of ribose and desoxyribose nucleic acid reaches a maximum per cell at, or just before, actual cell multiplication could be detected. It has been

found for both strains that the RNA increase over the initial amount present per cell lies between 5 and 10, while the maximum DNA increase has been ap-
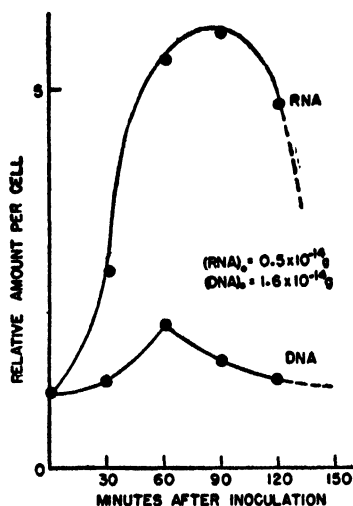
Figure 3. Changes in the average nucleic acid content of *E. coli*, strain B, during growth. Growth in synthetic medium.
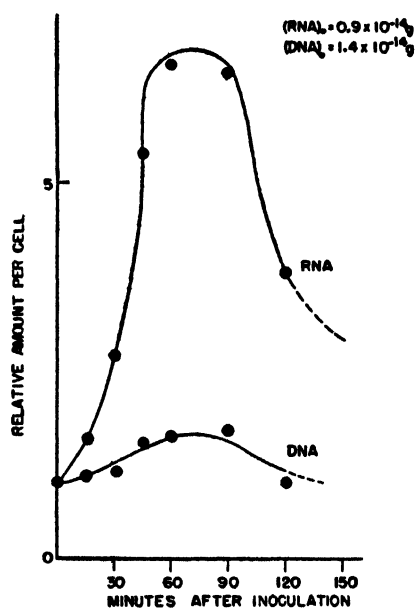
Figure 4. Changes in the average nucleic acid content of *E. coli*, strain B, during growth. Growth in broth.

proximately 2 in every case. It should be noted however, that cells of the B/r strain contain about 3 to 4 times the amount of DNA that the B strain cells contain. Of considerable interest also is the fall in DNA content of the B/r cells

following the onset of the division. When division has commenced, the quantity of nucleic acid per cell in both strains falls and approaches that initially present
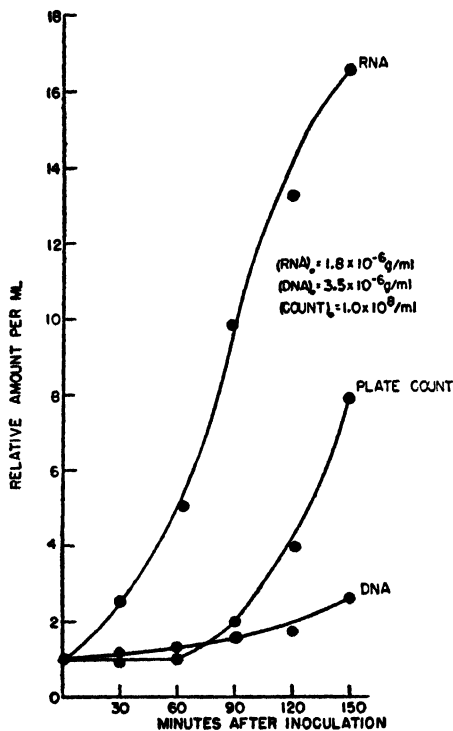
Figure 5. Synthesis of nucleic acid by *E. coli*, strain B/r.
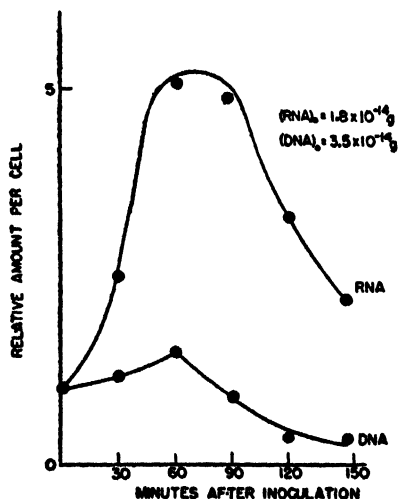
Figure 6. Changes in the nucleic acid content of cells of *E. coli*, strain B/r, during growth.

in the cell, except in the case of the B/r strain where the DNA content reaches a level approximately one-fourth that initially present.

As additional confirmation of the synthesis during the lag period two experiments were performed with the B strain using the uptake of P^{32} as an indicator

TABLE 1

Distribution of radiophosphorus taken up during the lag phase*

FRACTION	SAMPLING TIME IN MINUTES AFTER INOCULATION					
	0	10	20	30	40	60
A (acid sol.)	566,125†	573,375	515,062	470,025	402,600	‡
B (EtOH sol.) .	2,600	4,560	16,000	21,300	33,100	47,100
C (RNA)... ..	2,560	14,800	48,400	86,000	132,000	271,000
D (DNA)..	3,200	2,700	5,340	9,800	14,200	33,800
E (insoluble).. .. .	290	660	1,240	6,850	5,710	6,760
Totals....	574,775	596,095	586,042	593,975	587,610	—

* The plate count remained constant throughout this experiment.
† Counts per minute per fraction.
‡ Sample lost.
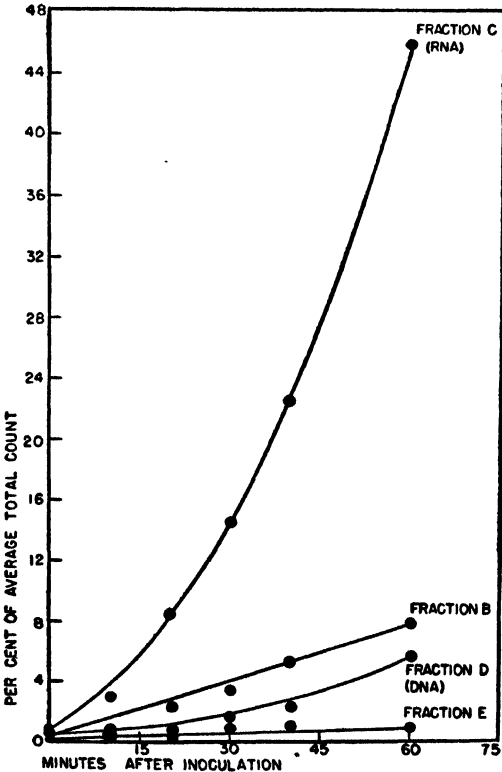
Figure 7. Distribution of radiophosphorus taken up during the lag phase.

of the nucleic acid synthesis. The gross counts of the various fractions of one of these experiments is shown in table 1, and the distribution among the fractions,

expressed as a percentage of the average total counts, is shown graphically in figure 7. The amount of phosphorus in each fraction was too low for satisfactory microchemical estimation; hence, specific activity of the several fractions could not be determined and the results are expressed as total counts per minute in each fraction. The rapid incorporation into the RNA fraction and the heavy labeling of this fraction are evident. After 60 minutes of incubation approximately 75 per cent of all radiophosphorus incorporated has been incorporated into the RNA fraction.

A note should be made of the small amount of P^{32} in all fractions at zero time, although this sample was removed from the culture and precipitated within 30 seconds of inoculation. This may represent contamination of the fractions with inorganic P^{32}, or it may result from a very rapid rate of incorporation of the isotope into organic compounds. On the assumption that the zero incubation time values represent inorganic P^{32} contamination, subsequent determinations should be corrected accordingly.

DISCUSSION

The results described above confirm and enlarge upon the observations of Malmgren and Heden with *E. coli* (1947c). The significance of the large increment in ribose nucleic acid during the lag phase is not known but appears to be connected with the protoplasmic and protein synthesis that occurs after cell division has begun. That ribose nucleic acid is involved in the synthesis of protein has been inferred by Caspersson (1947).

The syntheses observed occur in the phase of culture growth referred to as the phase of "physiological youth." It is during this period that many maxima of chemical activity are observed in bacterial cultures. Maximum activity per cell for the production of heat, carbon dioxide, ammonia, and hydrogen sulfide, as well as a maximum consumption of oxygen, has been found to occur at this time. Cells from cultures in this stage have also been found to possess other characteristics that distinguish them, such as increased size and ability to take up stains, increased ability to support bacteriophage multiplication, increased susceptibility to heat and cold, increased susceptibility to ultraviolet light; lowered agglutinability to both antisera and salts; and lowered electrophoretic mobility (Winslow and Walker, 1939).

Information on the connection between these activities and nucleic acid is limited but in most instances some connection has been intimated by previous work. The importance of nucleotides in metabolism and the intimate association of nucleic acids and staining reactions are well known. More recently evidence has been shown of a connection between adaptive enzyme formation and nucleic acids (Spiegelman, 1948) and between bacteriophage synthesis and nucleic acids (Price, 1948).

The differences observed between the B and the B/r strains remain to be clarified. It may be possible to resolve the radiation resistance phenomenon of the B/r strain about these differences. The difference in desoxyribonucleic acid pattern may also be of significance in the delay in expressing mutant characters

observed with this strain (Demerec, 1946; Newcombe, 1948), although such a delay has also been observed with the B strain (Demerec and Latarjet, 1946; Demerec, 1946).

SUMMARY

Cultures of *Escherichia coli*, strains B and B/r, have been examined for ribose and desoxyribose nucleic acid during the early phases of growth. A large amount of ribose nucleic acid is synthesized during the lag period. This synthesis has been confirmed with *E. coli* B by use of radiophosphorous as an indicator of nucleic acid content. It has been found that cells of the B/r strain contain 3 to 4 times the amount of desoxyribose nucleic acid that the cells of the B strain contain. A difference between the cells of the two strains following inoculation into fresh medium has been observed.

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AEROBIC SPOREFORMING BACTERIA CAPABLE OF GROWTH AT HIGH TEMPERATURES[1]

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The collection of 621 strains of aerobic mesophilic sporeforming bacteria studied by Smith, Gordon, and Clark (1946) included 8 strains that grew slowly, if at all, at 28 C and required a higher temperature than the others for maximum growth. They were closely related and as a group were distinguishable in several respects from the other species. Pending the investigation of more strains the 8 were assigned to the species *Bacillus coagulans* Hammer (1915), with which *Bacillus thermoacidurans* Berry (1933) is in synonymy.

Our interest in acquiring and examining additional strains of this poorly represented species was twofold. In the first place, a larger number was needed to give a more complete characterization of the species, for only through the study of an adequate number of strains can a reliable description of a species be prepared. Also, as an apparent intermediate between the mesophilic and thermophilic sporeformers, *Bacillus coagulans* seemed from the data available to be an inviting preliminary step toward the study of the so-called thermophilic group.

Thermophilic bacteria have been characterized by different workers in a variety of ways. Prickett (1928) ably summarized the various definitions and accepted the following one of Cameron and Esty (1926): Cultures growing at 55 C but not at 37 C were classified as obligate thermophiles, and those growing at both 55 C and 37 C, as facultative thermophiles. According to this definition none of the strains studied by Smith, Gordon, and Clark (1946) were obligate thermophiles; but 7 of the 8 strains of *Bacillus coagulans* and a few of *Bacillus subtilis* were facultative.

Approximately 60 different species of aerobic thermophilic sporeforming bacteria have been named and described. In the sixth edition of *Bergey's Manual* (Breed *et al.*, 1948, p. 707), Smith offered this apology: "The data on the species of this group are so meager that it is not possible to offer a rational system of classification. Many of the characters used for separating the various species are probably as variable in this group as they have been found to be in the mesophilic group. Lacking a knowledge of the limits of variability and lacking other pertinent data, the present arrangement is regarded as temporary only."

It was the purpose of this work, therefore, to study *Bacillus coagulans* more thoroughly, to characterize the species growing at still higher temperatures, and,

if possible, to show a relationship between the mesophilic and thermophilic groups.

<div align="center">MEDIA AND METHODS</div>

Although many of the media used in this work are common in most laboratories, some may not be well known. For the sake of completeness, all media used are listed below:

Nutrient agar. Peptone, 5 g; beef extract, 3 g; agar, 15 g; distilled water, 1,000 ml; pH 7.0.

Glucose agar. Nutrient agar with 1 per cent glucose.

Nutrient broth. Peptone, 5 g; beef extract, 3 g; distilled water, 1,000 ml; pH 7.0.

NaCl broth. Nutrient broth with 5 per cent NaCl.

Glucose asparagine agar. Asparagine, 0.5 g; K_2HPO_4, 0.5 g; agar, 15 g; distilled water, 1,000 ml; glucose, 10 g; pH 7.0 (a modification of Conn's glycerol asparaginate agar—Conn, 1921).

Tomato yeast milk. Difco litmus milk, 1,000 ml; yeast extract, 5 g; tomato juice, 100 ml. The pH of the tomato juice was adjusted to 7.0 before its addition to the other ingredients (Tittsler, 1944).

Citrate agar. NaCl, 1.0 g; $MgSO_4$, 0.2 g; $(NH_4)_2HPO_4$, 1.0 g; KH_2PO_4, 1.0 g; Na-citrate, 2.0 g; agar, 15 g; distilled water, 1,000 ml; pH 7.0; 0.04 per cent phenol red solution, 20 ml (Koser, 1924).

Soybean agar. One hundred grams of yellow soybeans were autoclaved for 1 hour at 121 C with 1,200 ml of distilled water and filtered through paper. The broth was made up to 1,000 ml; 15 g of agar were added and dissolved; the pH was adjusted to 6.8; and the medium was tubed and autoclaved for 20 minutes (Tsen and Sung, 1932).

Proteose peptone acid agar. Proteose peptone, 5 g; yeast extract, 5 g; glucose 5 g; K_2HPO_4, 4 g; distilled water, 500 ml; pH 5.0. An equal volume of 2 per cent agar, pH 5.0, was prepared and autoclaved separately. After sterilization the two solutions were cooled to about 50 C, mixed, tubed aseptically, and slanted (Stern, Hegarty, and Williams, 1942).

Stock culture agar. Difco stock culture agar (Ayers and Johnson, 1924) solidified by the addition of 7.5 g of agar per 1,000 ml.

Microscopic examination. Sixteen- to 18-hour cultures on nutrient agar were smeared on a clean slide, air-dried, and lightly stained with aqueous fuchsin (0.5 ml of a saturated alcoholic solution of basic fuchsin in 100 ml of distilled water). The vegetative cells were measured with an ocular micrometer. Another slide was stained by the gram method. Sixteen- to 18-hour cultures on nutrient agar were also observed for motility. After 2 or more days' incubation the cultures were again smeared and stained with aqueous fuchsin and the spores measured.

Temperatures for growth. To determine growth at various temperatures inoculated nutrient agar slants were immediately placed in a water bath of the desired temperature. After they had reached equilibrium with the bath they were

transferred to a similar water bath kept inside a constant temperature incubator. Observations for growth were made after 1 to 3 days at the higher temperatures (45 C to 70 C). At 37 C and below the cultures were held for 7 days.

Hydrolysis of starch. The cultures were streaked on plates of starch agar[2] and incubated for 2 or 3 days at the proper temperature. Hydrolysis of the starch was determined by flooding the plates with 95 per cent alcohol (Kellerman and McBeth, 1912). A clear zone underneath and around the growth gave the measure of hydrolysis, while unchanged starch became white and opaque. If no hydrolysis was observed another plate was inoculated and incubated for a longer period (5 to 7 days). The spreading of some cultures over the entire plate was prevented by drying the plates at 28 C for 3 to 5 days before inoculation.

Hydrolysis of gelatin. Plates of nutrient agar with 0.4 per cent gelatin were streaked once across and incubated at a suitable temperature. After an incubation of 3 to 5 days the plates were moistened with 8 to 10 ml of the following solution: $HgCl_2$, 15 g; concentrated HCl, 20 ml; distilled water, 100 ml (Frazier, 1926). The extent of hydrolysis of gelatin was indicated by a clear zone underneath and around the growth in contrast to the white opaque precipitate of the unchanged gelatin. As in the case of the starch agar plates, the spreading of some cultures had to be checked by drying the plates at 28 C for several days before inoculation.

Production of acetylmethylcarbinol. Five-ml amounts of the following broth were sterilized in 18-mm tubes: proteose peptone, 7 g; NaCl, 5 g; glucose, 5 g; distilled water, 1,000 ml. A series of tubes for each culture was inoculated, incubated at the proper temperature, and tested for acetylmethylcarbinol at 3, 5, and 7 days (Smith, Gordon, and Clark, 1946). The test was made by mixing the culture with an equal volume of 40 per cent NaOH and adding a few crystals of creatine from a knife point. The appearance of a red color after the culture stood 15 to 30 minutes demonstrated the presence of acetylmethylcarbinol (O'Meara, 1931).

pH of glucose broth cultures. Before the 7-day-old cultures were tested for acetylmethylcarbinol, about 1 ml was withdrawn and the pH determined by the colorimetric method.

Fermentation of carbohydrates. A 10 per cent aqueous solution of each carbohydrate to be tested was sterilized by autoclaving or filtration through an L2 Pasteur-Chamberland filter. One-half-ml amounts of the carbohydrate solution were then mixed aseptically with 5-ml sterile portions of an organic nitrogen and an inorganic nitrogen base. The organic nitrogen base used was nutrient agar with 15 ml per liter of a 0.04 per cent solution of bromcresol purple as indicator. The inorganic nitrogen base, a modification of the medium of Ayers, Rupp, and Johnson (1919), had the following composition: $(NH_4)_2HPO_4$, 1.0 g; KCl, 0.2 g; $MgSO_4$, 0.2 g; agar, 15 g; distilled water, 1,000 ml. The pH of the medium was adjusted to 7.0, and 15 ml of a 0.04 per cent solution of bromcresol purple were

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[2] Nutrient agar with 1 per cent potato starch added. If filtration of the agar is necessary, it must be done before the addition of the starch.

added. Both the inorganic and organic nitrogen media were inoculated, incubated at the proper temperature, and observed for acid production at 3, 5, 7, and 10 days.

Reduction of nitrate to nitrite. The method adopted for this determination was described in the *Manual of Methods* (1947). The cultures were grown in nutrient broth containing 0.1 per cent KNO_3 and incubated at suitable temperatures. After 1 and 3 days, 1 ml of the broth culture was mixed with 3 drops of each of the following two solutions: (1) sulfanilic acid, 8 g; 5 N acetic acid (1 part glacial acetic acid to 2.5 parts of water), 1,000 ml; (2) dimethylalphanaphthylamine, 6 ml; 5 N acetic acid, 1,000 ml. The appearance of a deep red color or a heavy yellowish precipitate demonstrated the presence of nitrite.

Isolation of cultures. The following technique was usually employed in this laboratory for the isolation of thermophilic aerobic cultures: Small amounts of milk, cheese, cream, soil, or silage were added to tubes of sterile nutrient broth, heated to 85 C, and held for 5 to 10 minutes. After the preliminary heating the tubes were quickly cooled to 57 C and incubated overnight in a water bath at the latter temperature. The broth cultures were then plated on nutrient agar and incubated at 45 C or 57 C. Isolated colonies representative of the types present on the plates were selected and transferred to nutrient agar slants.

This method was frequently modified in one or more of the following ways: The material was suspended in glucose or meat infusion broth; 100 C for 5 minutes was used instead of 85 C to destroy all vegetative forms; after the first heating the broth suspensions were incubated at 55 C, 60 C, 62 C, or 65 C for 24 or 48 hours; and the final plating was made on glucose or starch agar instead of on nutrient agar.

COMPARATIVE STUDY OF CULTURES

Sources and identification of cultures. In spite of widespread requests for named strains of aerobic thermophilic sporeforming bacteria sent to many laboratories in this country and Europe, only 33 named cultures were received. This was very surprising to the writers in view of the number described in the literature and the ease with which sporeforming bacteria may be kept alive for years without any attention. The remaining 183 strains of the collection of 216 were unidentified isolations. Many of these, supplied by other laboratories, were obtained from spoiled canned foods. In addition, isolations were made by the writers from soil, silage, milk, and milk products. If any of the cultures so isolated were found to belong to a species heretofore considered mesophilic, i.e., *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus circulans*, *Bacillus macerans*, *Bacillus brevis*, etc., they were discarded. Of the unnamed cultures supplied by other laboratories in response to requests for thermophilic strains, 39 per cent belong to the above-mentioned species or to species with even lower temperature ranges of growth.

All strains of the collection were studied microscopically, macroscopically, and physiologically by as many means as time would permit. Of the many characteristics determined, the most reliable were selected to describe and separate each species. Identification of a strain was not intended to depend on one or two,

but rather on a group of characters or on a species' pattern. If the reactions and properties of a strain conformed in the main, not necessarily in every detail, to a species' pattern, the strain was considered a member of the species. In this way allowance was made for the natural variation of microbial cultures.

The identification of the cultures of this collection is summarized in table 1. A comparative examination of the 33 named cultures showed that with one exception they belonged to only two distinct groups. The earliest named and described species in one group was *Bacillus stearothermophilus* Donk (1920)

TABLE 1

Allocation of 206* cultures assembled for the study of the aerobic thermophilic sporeforming bacteria

SOURCES OF CULTURES STUDIED	NUMBERS AND IDENTIFICATIONS OF CULTURES								
	B stearo- thermo- philus	*B. coag- ulans*	*B. sub- tilis*	*B. circu- lans* complex	*B. mac- erans*	*B. pu- milus*	*B. cereus*	*B. brevis*	*B. sphae- ricus*
Named strains† ...	8	24				1			
Isolations from other laboratories*									
Canned foods .	49	21	17	6	4	2	2	2	1
Milk and milk products	5		2	5					
Grass cuttings	8		4						
Silage		1							
Isolations by the writers									
Milk and milk products . .	5	21							
Soil and silage	12	6							
Totals	87	73	23	11	4	3	2	2	1

* In addition, 10 mesophilic cultures that were not easily recognizable were put aside for future study.

† These strains bore a variety of names. See tables 3 and 4.

and in the other *Bacillus coagulans* Hammer (1915). The one culture not falling into either of these two groups and labeled *Bacillus thermophilus* was found to be a typical strain of *Bacillus pumilus* Gottheil.

The lack of more distinct groups, or species, among the named strains was also surprisingly exhibited by the 183 unidentified isolations. Of these, 128 cultures proved to be either *Bacillus stearothermophilus* or *Bacillus coagulans;* 45 were recognized as members of species previously regarded as mesophilic, and 10 could not be readily identified as belonging to any of the species listed in table 1. All of these 10 grew well at 28 C; 9 grew at 45 C but not at 50 C; and the remaining one, at 50 C but not at 55°C. As they apparently were mesophilic rather than thermophilic, the time required for their identification was not given during this study.

Temperature relations of cultures. The ability to grow at temperatures of 28 C to 70 C inclusive was determined for a number of strains of each species as indicated in table 2. The results are arranged to show the decrease in the upper limits of growth of the various species. Approximately one-half of the cultures of *Bacillus stearothermophilus* grew at 70 C and all grew at 65 C. All strains of *Bacillus coagulans* failed to grow at 65 C; all strains of *Bacillus subtilis*, *Bacillus brevis*, and *Bacillus circulans* failed at 60 C; all strains of *Bacillus pumilus* and *Bacillus macerans*, at 55 C; and all of *Bacillus cereus* and *Bacillus sphaericus*, at 50 C. With the exception of *Bacillus stearothermophilus* and *Bacillus coagulans*, all cultures of the species listed above grew at 28 C.

It is apparent that the temperatures of 55 C and 37 C selected by Cameron and Esty (1926) for the classification of thermophilic sporeformers cannot be

TABLE 2

Growth temperatures of species of aerobic sporeforming bacteria represented in this collection

SPECIES	NUMBER OF CULTURES	NUMBER OF CULTURES GROWING AT VARIOUS TEMPERATURES*								
		28C	33C	37C	45C	50C	55C	60C	65C	70C
Bacillus stearothermophilus ...	87	0	10	73	81	87	87	87	87	45
Bacillus coagulans	73	53	73	73	73	72	66	23	0	
Bacillus subtilis	154	154	154	154	150	105	17	0		
Bacillus brevis	57	57	57	57	38	16	7	0		
Bacillus circulans	55	55	55	51	18	6	1	0		
Bacillus pumilus.	65	65	65	65	64	43	0			
Bacillus macerans........... ..	13	13	13	13	13	9	0			
Bacillus cereus.	50	50	50	50	23	0				
Bacillus sphaericus	42	42	42	42	15	0				

* Water bath temperatures were used in all cases except for 28 C.

used to differentiate species. Of the higher temperatures, only 65 C separated species. At this point all the strains of *Bacillus stearothermophilus* grew and none of *Bacillus coagulans* did. Approximately one-third of the strains of the latter species grew at 60 C, making the separation of *Bacillus coagulans* from *Bacillus stearothermophilus* on the basis of temperature a procedure involving not more than a 5-degree margin.

In addition to the comparison of the maximum temperatures permitting growth of the various species, table 2 shows the great differences among strains of a species in respect to their maximum temperatures of growth. Although 17 cultures of *Bacillus subtilis*, for example, grew at 55 C, 4 failed at 45 C. From a taxonomic standpoint, therefore, little reliance can be put on a strain's maximum temperature of growth for its species identification.

In this connection the question might be raised as to whether those strains of a species having a low maximum temperature might be acclimatized to grow at a higher temperature. A few unsuccessful attempts made by the present writers along this line and the work of Casman and Rettger (1933) indicate that the maximum temperature of growth of a culture is not likely to change. In experi-

ments of a year's duration Casman and Rettger attempted to induce members of the "subtilis group" to grow at higher temperatures. When cultivated on agar slants their strain F of *Bacillus subtilis*, for example, grew at a maximum temperature of 59 to 60 C and strain T at a maximum of 49 to 50 C, or 10 C lower than strain F. Neither strain F nor strain T could be adapted to grow at higher temperatures.

BACILLUS STEAROTHERMOPHILUS DONK (EMEND.)

From the detailed study of 87 cultures an emended description of *Bacillus stearothermophilus* Donk (1920) has been prepared and is given below. The source of each culture studied and what is known of its history are given in table 3. The six type cultures listed represent only three different strains at the most, owing to duplications. Three cultures bearing names of two other species were identical with *Bacillus stearothermophilus*. Of the two species only *Bacillus kaustophilus* Prickett (1928) can be considered in synonymy. The status of *Bacillus calidolactis* Hussong and Hammer (1928) is in doubt because two cultures with this name were found to be *Bacillus stearothermophilus* and three others were *Bacillus coagulans* Hammer (1915). Hussong and Hammer's description of *Bacillus calidolactis* offers no clue to the identity of the original culture. In addition, 79 unnamed cultures from canned foods, starch and sugar solutions, milk, soil, and compost were included. The cultures from the latter source were isolated by Webley (1947).

Vegetative rods. Most of the cultures of this species formed vegetative cells measuring 0.9 to 1.0 μ by 2.5 to 3.5 μ. The cells of a few cultures were less than 0.9 μ in width and in one instance (no. 77) as small as 0.6 μ. Cells from 2.0 to 5.0 μ long and filaments were occasionally observed. Gram's reaction was variable. All cultures were motile.

Sporangia. Sporebearing cells were definitely swollen.

Spores. Mature spores measured 1.0 to 1.2 μ by 1.5 to 2.2 μ. They were oval and subterminal to terminal, rarely central.

Macroscopic appearance. The macroscopic appearance of the cultures of this species varied so greatly that it was valueless in the recognition of the species. The growth on nutrient agar ranged from thin, scant, rough, and nonspreading to abundant, opaque, smooth, and spreading.

Temperatures of growth. All 87 strains grew from 50 C to 65 C inclusive, and 45 (51 per cent) grew at 70 C, water bath temperatures (see table 2). Growth at these high temperatures is apparently a stable character because strains growing at 65 C and 70 C when first examined still grew at these temperatures after 4 years' storage at 28 C. At the lower temperatures, 6 failed to grow at 45 C; 14 at 37 C; 77 at 33 C; and all failed at 28 C. After 4 years' storage, however, many strains grew at lower temperatures than at the beginning. As a result, growth at 65 C is considered a fairly reliable character of this species, whereas growth at the lower temperatures is too variable to be of use.

Growth on proteose peptone acid agar, pH 5.0. All strains were negative.

Curd in tomato yeast milk. None of the strains formed a curd in this enriched

TABLE 3

TABLE 3
Cultures identified as Bacillus stearothermophilus Donk

LABORATORY NUMBER	SOURCE AND HISTORY
	Named cultures
17, 19	B. stearothermophilus; Curran;* NCA* (1518, 1503)
85	B. stearothermophilus; ATCC* (7954); NCA (1503)
91, 92	B. stearothermophilus; NCA (1503, 1518)
2004	B. stearothermophilus; Brooks;* ACC* (CDPI)
15, 16	B. calidolactis; Curran; Iowa State College†
81	B. kaustophilus; Yale* (BD53)
	Isolations from canned foods
18, 20	Curran; NCA (26, 7028)
28 to 33	ACC (7B, 21Y, unnumbered, 26A, 26B, 36A)
53, 59 to 63	NCA (T38, T28, T30, T34, 1792, T9)
64 to 70	NCA (T6, 4298, 37, Avon X-1, 2116, 4109, T36)
71 to 78	NCA (T33, T13, 1356, 1549, 1805, 4172, 4103, Avon M-1)
79, 80, 82	NCA (4208, 2027, HB)
117, 127	NCA (1373, 1820)
132, 141	NCA (2548-11, 1168)
188, 189	Curran; ACC (C_1P_4, C_2P_3)
194 to 197	NCA (1889, 2263-1, 2263-2, 2263-3)
	Isolations from milk
34 to 37, 39	Speck* (TNA 25, TH 63, TH 54, TNAI, LTI)
86 to 90	Writers' collection
	Isolations from soil
93 to 95	Writers' collection
106 to 110	Writers' collection
152 to 155	Writers' collection
	Isolations from compost
158 to 160	Webley* (ATT, BTT, DTT)
164 to 168	Webley (HTT, JTT, KTT, LTT, NTT)
	Isolations from starch and sugar solutions
137	NCA (11555)
146 to 150	NCA (C-1194, C-1225 no. 3, C-1235 no. 1, C-1235 no. 4, C-1235 no. 5)

* The writers wish to thank the following investigators who kindly donated cultures for this study: H. R. Curran,. U. S. Department of Agriculture, Washington, D. C.; C. W. Bohrer and J. Yesair, National Canners Association (NCA), Washington, D. C.; R. F. Brooks, M. W. Yale, and C. S. Pederson, New York (Geneva) Agricultural Experiment Station; R. W. Pilcher, American Can Company (ACC), Maywood, Illinois; M. L. Speck, Sealtest Research Laboratories, Baltimore, Maryland; D. M. Webley, University of Aberdeen, Scotland; D. P. Renco, Instituto Spermentale di Caseficio di Lodi, Milan, Italy; E. Olsen, Danmarks Tekniske Hojskole, Copenhagen, Denmark; Elizabeth McCoy, University of Wisconsin, Madison, Wisconsin.

† For the allocation of other cultures bearing this name see nos. 2006 to 2008 listed under *Bacillus coagulans.*

milk. Although 10 of the 87 strains showed a small amount of acid or reduction, the rest produced no change at all during 3 days' incubation at 45 C or 50 C.

Growth on soybean, stock culture, and glucose agars. With growth on nutrient agar as a basis for comparison, 85 of the 87 strains were inhibited on soybean agar; 81, on stock culture agar; and 77, on glucose agar. In every case when there was no inhibition on one of the three mediums, there was definite inhibition on the other two. This fact, therefore, made the combined results on the three agars a dependable character for the description of the species.

Growth in 5 per cent NaCl broth. All strains were negative.

Production of acetylmethylcarbinol. All strains were negative.

Hydrolysis of starch. All strains were positive.

Reduction of nitrate to nitrite. Eighty-eight per cent of the strains reduced nitrate to nitrite, most of them giving a strongly positive reaction.

Hydrolysis of gelatin. Ninety-four per cent of the strains exhibited a wide zone of hydrolysis on gelatin agar plates (exceptions, nos. 34, 36, 39, 141, and 146).

pH of glucose broth. Considerable acidity in glucose broth was produced by 82 strains after 7 days. Ninety per cent were less than pH 5.5 and 40 per cent were less than pH 5.0. The growth of 5 strains of this group, however, was greatly inhibited apparently by a very small amount of acid from the glucose, the pH after 7 days being not lower than 6.6.

Fermentation studies. In a preliminary study of a small number of strains of this group the fermentation of a variety of carbohydrates was tested. As the results were extremely variable and correlated with no other character, only the fermentation of glucose, xylose, and arabinose, with both an organic and an inorganic nitrogen base, was completely studied. All of the 87 strains formed acid from glucose in nutrient agar, and all but 9 were positive on the ammonium phosphate medium. Xylose was fermented by 45 per cent of the strains and arabinose by 24 per cent when nutrient agar was used. With the ammonium phosphate medium fewer strains formed acid, probably because of their inability to use inorganic nitrogen.

Growth on citrate agar. Only 3 (nos. 141, 148, and 149) of the 87 strains utilized citrate as a source of carbon.

Growth on glucose asparagine agar. Forty-one per cent of the strains grew slightly to moderately on this medium.

BACILLUS COAGULANS HAMMER (EMEND.)

Although an emended description of *Bacillus coagulans* Hammer (1915) was given by Smith, Gordon, and Clark (1946), the study of a greater number of cultures has shown the need of several modifications. It is now believed that these changes and the additional characters listed below will be sufficient to define the species. Eleven of the 73 cultures on which the species' description presented here is based were type cultures. Two cultures of *Bacillus thermoacidificans* Renco (1942), 8 cultures of *Bacillus thermoacidurans* Berry (1933), 2 of *Lactobacillus cereale* Olsen (1944), and 3 of *Bacillus calidolactis* Hussong and Hammer (1928) were found to be *Bacillus coagulans*. The first three species are considered

to be in synonymy with *Bacillus coagulans*. The position of the fourth species remains in doubt because two other cultures labeled *Bacillus calidolactis* proved to be *Bacillus stearothermophilus*. In addition to the named strains there were 49

TABLE 4

Cultures identified as Bacillus coagulans Hammer

LABORATORY NUMBER	SOURCE AND HISTORY
	Named cultures
13	*B. coagulans;* Curran* (195); Iowa State College
609, 770, 784	*B coagulans;* writers' collection
795 to 798	*B. coagulans;* writers' collection
2006 to 2008	*B. calidolactis;* Brooks;* Iowa State College (C1, 01, sp.)†
186	*B. thermoacidificans;* Renco*
2011	*B. thermoacidificans;* Olsen;* Renco
21 to 27	*B. thermoacidurans;* ACC* (94S, 81S, 8T, 4E, 6, 43P, 78G)
2005	*B. thermoacidurans;* Brooks;* ACC (20)
2012, 2013	*Lactobacillus cereale;* Olsen (U, B)
	Isolations from canned foods
14	Curran; NCA* (1460)
54 to 58	NCA (4167, 1264, 52-240, 4578, 4110)
114, 115, 121	NCA (1215, 1264, 1460)
126, 134	NCA (1734, 848)
138 to 145	NCA (518, 698, 880, 1150, 11878, 2, 4)
198, 200	NCA (C-1655, C-1657)
2016	Pederson*
	Isolations from cream
96 to 105	Writers' collection
	Isolations from cheese
169 to 176	Writers' collection
190 to 192	Writers' collection
	Isolations from silage
83	McCoy;* Pan (E)
177, 178	Writers' collection
181 to 184	Writers' collection

* See the first footnote of table 3.

† For the allocation of other cultures bearing this name see nos. 15 and 16 listed under *Bacillus stearothermophilus.*

isolates from canned foods, cream, cheese, and silage. The cultures from silage included strain E of Pan, Peterson, and Johnson (1940). (See table 4.)

Vegetative rods. Most of the cells of cultures of this species measured 0.6 to 1.0 μ by 2.5 to 5 μ. Variations from 0.5 to 1.2 μ by 2 to 6 μ to filaments were recorded.

All but one (no. 25) of the 73 cultures were motile. Gram's reaction was usually positive, although a few variable strains were observed.

Sporangia. In some cases the sporangia were definitely swollen; in others there was no enlargement.

Spores. Mature spores were oval, thin-walled, subcentral to terminal, and measured 0.9 to 1.0 μ by 1.2 to 1.5 μ, with occasional variation from 0.8 to 1.1 μ by 1.2 to 2.0 μ.

Macroscopic appearance. The growth of cultures of this species on nutrient agar slants was noncharacteristic. It varied from thin, smooth, spreading, and translucent to moderate, rough, patchy, and opaque. Growth on agar with a fermentable carbohydrate was usually thick, soft, and moist.

Temperatures of growth. Fifty-three of the 73 cultures grew at 28 C; all grew from 33 C to 45 C; all but one at 50 C; and none at 65 C (see table 2).

Growth on proteose peptone acid agar, pH 5.0. All cultures grew well.

Curd in tomato yeast milk. This enriched milk was curdled by 97 per cent of the cultures in 1 to 3 days at 45 C (exceptions, nos. 200 and 2006).

Growth on soybean, stock culture, and glucose agars. All of the cultures grew as well or better on soybean and glucose agars than on nutrient agar. With the exception of 4 cultures (nos. 139, 140, 142, and 2006) stock culture agar supported as good or heavier growth.

Growth in 5 per cent NaCl broth. All cultures failed to grow.

Production of acetylmethylcarbinol. Seventy per cent of the cultures were positive. Serial transfers in V-P broth and on soybean agar failed to induce the formation of acetoin by the negative strains.

Hydrolysis of starch. All but two of the cultures (nos. 139 and 178) hydrolyzed starch.

Reduction of nitrate to nitrite. Twenty-two per cent of the cultures reduced nitrate to nitrite.

Hydrolysis of gelatin. Gelatin was hydrolyzed only slightly, if at all, by 72 of the 73 cultures (exception, no. 115).

pH of V-P broth. With 2 exceptions (nos. 797 and 2006) the pH of these cultures in glucose broth at 7 days was less than 5.0, ranging from 4.4 to 4.8.

Fermentation studies. As in the case of *Bacillus stearothermophilus*, the fermentation of most of the carbohydrates tested was variable and furnished little assistance in defining the species. The 73 cultures of *Bacillus coagulans* formed acid from glucose in nutrient agar and all but one (no. 104) were positive in the ammonium phosphate medium. Xylose was fermented by 79 per cent of the cultures when nutrient agar was used and by 65 per cent with the ammonium phosphate medium. Arabinose was hydrolyzed by 58 per cent of the cultures in both mediums.

Growth on citrate agar. Seventy-one of the 73 cultures failed to grow on citrate agar (exceptions, nos. 142 and 143).

Growth on glucose asparagine agar. Seventy-two of the 73 cultures grew only slightly, if at all, on this agar (exception, no. 905).

SEPARATION OF CLOSELY RELATED SPECIES

In the present work on the aerobic sporeforming bacteria able to grow at high temperatures it was obvious that there were five species that might be confused with one another, i.e., *Bacillus stearothermophilus* Donk, *Bacillus coagulans* Hammer, *Bacillus subtilis* Cohn emend. Prazmowski, *Bacillus pumilus* Gottheil, and *Bacillus circulans* Jordan emend. Ford. The remaining species appearing in table 1 and represented in this collection by only a very few cultures are distinct and recognizable, and no additions were made to their definitions as given by Smith, Gordon, and Clark (1946).

According to the emended descriptions of *Bacillus stearothermophilus* and *Bacillus coagulans* offered above, the principal means of separating typical cultures of these two species were as follows: *Bacillus stearothermophilus* grew at 65 C and hydrolyzed gelatin moderately to strongly; its growth was inhibited on glucose, soybean, and stock culture agars. On the other hand, *Bacillus coagulans* did not grow at 65 C, hydrolyzed gelatin weakly, if at all, and grew as well as or better on the 3 agars selected than on nutrient agar. *Bacillus coagulans* grew abundantly on proteose peptone acid agar, pH 5.0, and formed a curd in tomato yeast milk, whereas *Bacillus stearothermophilus* was negative in each case.

Further examination was made of the characteristics dividing *Bacillus subtilis* from *Bacillus coagulans*, a species with which it might be confused microscopically. The necessity for this re-examination was clearly indicated by the expansion of the number of cultures of *Bacillus coagulans* from 8 as reported by Smith, Gordon, and Clark (1946) to 73 and the subsequent increased knowledge of the species. *Bacillus pumilus* was included in this study because of its close relationship to *Bacillus subtilis*. In contrast to *Bacillus coagulans*, 159 cultures of *Bacillus subtilis* and 71 cultures of *Bacillus pumilus* grew in a 5 per cent concentration of NaCl and hydrolyzed gelatin, forming wide zones of hydrolysis on gelatin agar plates. All cultures of *Bacillus subtilis*, except nos. 263 and 979, and all of *Bacillus pumilus* utilized citrate and gave a strongly alkaline reaction on citrate agar. Glucose asparagine agar supported abundant growth by all but 6 of the 159 cultures of *Bacillus subtilis* (exceptions, nos. 263, 315, 714, 820, 941, and 979), and 3 of the 71 cultures of *Bacillus pumilus* (exceptions, nos. 710, 731, and 980). In V-P broth a pH of 5.0 to 8.6 was reached at 7 days by all cultures of *Bacillus subtilis* except no. 740 and by 69 of the 71 cultures of *Bacillus pumilus* (exceptions, nos. 620 and 638). *Bacillus pumilus* agreed with *Bacillus subtilis* in the preceding reactions but differed in its inability to hydrolyze starch or reduce nitrates to nitrites.

The swelling of the sporangia by the mature terminal spores of *Bacillus stearothermophilus* provided a reliable means of separating this species from *Bacillus subtilis* and *Bacillus pumilus*. The two latter species also varied from *Bacillus stearothermophilus* in several other ways, i.e., growth at 65 C, formation of acetoin, growth in 5 per cent NaCl, and utilization of citrate.

It is regretted that time did not permit further study of the *Bacillus circulans* complex and the establishment of dependable characters for the delineation of the species and for its separation from *Bacillus stearothermophilus* and *Bacillus*

coagulans. At present, growth at 65 C is the only means of differentiating *Bacillus stearothermophilus* from *Bacillus circulans*. Unfortunately, maximum temperature of growth cannot be used to divide the latter species from *Bacillus coagulans*. Pending future investigation, the inability of *Bacillus circulans* to grow on proteose peptone acid agar, pH 5.0, and to curdle tomato yeast milk is offered as a tentative basis for its separation from *Bacillus coagulans*. Although varying considerably within the *Bacillus circulans* complex, other characteristics were also helpful, such as the formation of thick-walled spores, a negative Gram's reaction, moderate to strong hydrolysis of gelatin, inability to grow on soybean agar, and inhibition of growth on glucose agar. As a result, strains of this complex must at present be identified on the basis of a large group of reactions and wide variations accepted as normal occurrences.

DISCUSSION

As previously stated by Smith, Gordon, and Clark (1946), the knowledge of the taxonomist is limited to his collection of strains. The small number of distinct groups among the named cultures and also among the unnamed isolations of this collection was unexpected. Although extensive efforts were made to obtain cultures from different workers and to make isolations from a variety of sources, the writers are reluctant to accept the collection of cultures described here as representing the entire group of aerobic thermophilic sporebearing bacteria. When more strains are studied, more species will undoubtedly be found and a modification of the classification presented here will perhaps be necessary.

The name, *Bacillus stearothermophilus*, given to the species of cultures growing at the highest temperatures is the name of the earliest defined species represented in this group. The literature was carefully searched for accounts of thermophilic aerobic sporeformers not present in this collection. The descriptions found, however, were inadequate and the original cultures unrecognizable. With none of the cultures predating *Bacillus stearothermophilus* at hand for comparative purposes, there is no choice at present but to assign this cumbersome name to the species. The appearance of earlier named cultures of this group for study or an appeal to the Judicial Commission of the Permanent International Committee on Bacteriological Nomenclature of the International Association of Microbiologists may result in the assignment of a more suitable name to the species.

The overlapping of temperature ranges of growth of the various species present in this collection as illustrated in table 2 clearly shows that temperatures of growth cannot be used for the separation of these species. Exception might be made in the case of *Bacillus stearothermophilus*, but the margin of difference is so small in this instance it is regarded with suspicion. How, then, shall the term "thermophilic" be applied? The writers suggest it be used in a general way to denote cultures capable of growth at 55 C in accordance with Cameron and Esty's definition, but never to describe species. It is recognized, of course, that a culture's ability to grow at 55 C is undoubtedly of more interest to investigators in the canning industry, for example, than its species identification and that the term "thermophilic" has recognized descriptive value. It is believed, therefore,

that the recommended restriction of the term to strains growing at 55 C offers the best solution available and that the avoidance of its use in the taxonomy of species will tend to lessen further confusion over the identity of species of the genus *Bacillus*.

Attention should be called again to the fact that, in high temperature incubators, evaporation from the medium, radiation from the heater, stratification of the air, etc., alter the actual temperature of the medium on which the microorganism is growing. The differential between the temperature of the medium and that shown by the incubator thermometer may be considerable, depending on the characteristics of the incubator. It is recommended, therefore, that for the determination of temperatures of growth above 37 C the cultures be incubated in water baths.

SUMMARY

A collection of 216 cultures was assembled for a taxonomic study of the aerobic sporeforming bacteria capable of growth at high temperatures. Only 33 named strains were obtained for comparative purposes, the remainder being unnamed isolations from canned foods, milk, milk products, soil, compost, and silage.

Thirty-nine per cent of the isolations donated by other laboratories and one of the named cultures were found to be strains of *Bacillus subtilis*, *Bacillus circulans*, or other species with even lower temperature limits of growth. The rest of the cultures, including 44 isolations by the writers, fell into two distinct groups, of which *Bacillus stearothermophilus* Donk and *Bacillus coagulans* Hammer (*Bacillus thermoacidurans* Berry) are representatives. Emended descriptions of these two species have been prepared.

Maximum temperature of growth (water bath determinations) was found to vary widely among the strains of individual species and, for this reason, cannot be used as a dividing character among species, except perhaps in the separation of all species thus far studied from *Bacillus stearothermophilus*.

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THE PROPERTIES OF FORMALDEHYDE-RESISTANT CORYNEBACTERIUM DIPHTHERIAE

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Recent literature has indicated a remarkable potentiality among bacterial species for variation in response to the development by them of resistance to various chemicals and antibiotic substances. Morton (1940), in an extensive review of the variations observed in *Corynebacterium diphtheriae*, has described certain morphological, colonial, and biochemical changes following the growth of this species in the presence of such substances as bile and bile salts, copper sulfate, magnesium sulfate, magnesium chloride, and copper nitrate, indicating a capacity on the part of this species to produce variations in response to certain chemical stimulations.

Preliminary experiments demonstrated that cultures of *C. diphtheriae* could develop tolerance to increasing quantities of formaldehyde and that certain changes in the organisms accompanied this resistance. The experiments were then extended to produce a marked increase in formaldehyde resistance in the culture, and the present paper is a description of the process and the observations made on the morphological, cultural, biochemical, virulent, and toxigenic properties of the resistant strain.

MATERIALS AND METHODS

The organism was a pure culture of *C. diphtheriae*, Park-Williams no. 8. Stock cultures were kept on heated blood agar slants, on Loeffler's medium, and in veal infusion broth in accordance with the specifications of Wadsworth (1947).

Organisms from 48-hour Loeffler's medium cultures were inoculated into veal infusion broth and, after incubation at 37 C for 48 hours, removed to the same medium containing 0.001 per cent formaldehyde. Successive 48-hour transfers were then made to media containing concentrations in a range from 0.001 to 0.2 per cent, the increment of increase with each passage being of the order of 0.001 up to 0.02 per cent formaldehyde, following which, increments were made at 0.02 per cent up to and including the flask containing a concentration of 0.2 per cent formaldehyde.

Subsequent to the development of resistance to each step in the foregoing series, as evidenced by growth at this concentration, the step was repeated, and the resistant organisms were transferred to a slant of Loeffler's medium containing no formaldehyde. Each of these cultures was subcultured in the same medium 35 successive times at 48-hour intervals. Control organisms were handled as above without the addition of formaldehyde to the media. Observations on morphology,

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colony type, biochemical reaction, virulence, and toxigenicity were made at regular intervals on both stages of the foregoing process.

Morphology, colony formation, pigmentation, and hemolytic action were observed on colonies grown on nutrient agar, tellurite agar, and blood agar. The biochemical properties were judged by tests for the fermentation of glucose, sucrose, maltose, and lactose, and by the capacity to reduce tellurite. Virulence was determined by the standard intradermal skin test according to Frobisher (1945), and toxin production after growth in veal infusion broth was measured by the Ramon flocculation test as outlined by Bayne-Jones (1924).

RESULTS

Throughout the entire study, the control cultures failed to grow at concentrations of formaldehyde of 0.006 per cent or above without having been brought up through the lesser concentrations beginning at 0.001 per cent formaldehyde. However, by the procedure described above, in which the bacteria were exposed on successive transfers to increasing concentrations of formaldehyde, the resistance was increased gradually so that eventually growth occurred in all concentrations including 0.1 per cent. Although this result was obtained in each of four successive trials, none of the resistant strains could be cultivated in the presence of 0.2 per cent formaldehyde.

Two possible explanations appear for this failure. The most probable is that the increment increase from 0.1 to 0.2 per cent was too great to permit survival without gradual exposure to lesser concentrations between these two values. Also, formaldehyde concentrations in a range of 0.2 to 0.4 per cent are known to bring about changes in toxin proteins, radical enough to detoxify them, and may represent an absolute limit to which the organisms are not able to adapt.

Morphological variation was evident at all states of the development of formaldehyde resistance. Whereas the control organism appeared as the typical short, club-shaped rod with palisade grouping and "snapping" division, the predominant morphological form in resistant cultures was threadlike or filamentous, with an exaggerated granular appearance. In addition, where these were transferred to Loeffler's medium without formaldehyde, there was a reversal of the gram stain reaction so that the organism appeared gram-negative with distinct gram-positive granules. These morphological changes seemed to be stable variations since they persisted for 35 successive transfers on Loeffler's medium containing no formaldehyde. It is also possible to correlate these changes with the acquisition of resistance since, on the return of the above-mentioned 35th passage culture to media containing formaldehyde, resistance was evident by growth equal to that originally observed. Similar filamentous forms with marked granules were observed by Grassett and Grassett (1930) in diphtheria bacilli grown in media containing bile or bile salts. The other feature of the resistant variant noted above, the modified staining reaction, which occurred only following the removal of the variant organisms to media containing no formaldehyde, is similar in this respect to formaldehyde-treated pneumococci which, according to Dubos (1938), underwent a gram stain reversal if the formaldehyde was removed by washing the organisms before staining.

The only other morphological variation observed in the present study was the appearance of spherical forms similar to those described by Grubb and Koser (1934) and Morton (1940). They occurred in cultures growing in media containing 0.007, 0.008, and 0.01 per cent formaldehyde, but disappeared on subculture to the Loeffler's medium containing no formaldehyde.

Coincident with the appearance of the filamentous cell forms, colonial changes were also evident. The resistant variant grew as a rough (R) colony with irregular margins and a flat, uneven, and granular surface. An inoculating needle passed through this colony caused fragmentation with pieces of growth clinging to the needle. When transferred to broth, these organisms grew as a pellicle with a slight sediment on the bottom, and saline suspensions were clumped spontaneously and settled out. In contrast, the control organism grew as a smooth (S) round colony with an even margin and a glistening, convex surface. The colonies had a smooth texture, so that no furrow occurred in the path of a needle passed through them; they grew in broth with a uniform turbidity, and formed stable saline suspensions. The rough forms appeared throughout the course of resistance development and had reached a maximum at formaldehyde concentrations of 0.01 per cent. This character was retained throughout subsequent transfers in the absence of formaldehyde. A similar colony variation produced by filamentous cell forms of C. diphtheriae has been reported by Neisser (1932), Hobby (1935), and Morton (1940), and in the present instance these changes seem to accompany formaldehyde resistance.

The biochemical reactions of the resistant form varied from those of the control organisms in several aspects. The latter fermented glucose and maltose with the production of acid, but sucrose and lactose were not acted upon. However, the organisms removed from cultures containing 0.001 per cent formaldehyde fermented sucrose beginning with the 14th transfer on Loeffler's medium and continued to show this property through the remainder of the 35 passages on this medium. Subcultures from 0.002 per cent formaldehyde showed this same fermentation of sucrose with the 7th transfer on Loeffler's medium, and cultures from concentrations of 0.003 per cent and above were capable of fermenting sucrose on the first subculture on medium without formaldehyde.

In all cases, the new fermentation property did not occur until the organisms were removed from the formaldehyde and was a stable feature of the resistant variant throughout the study. Although Morton (1940) has indicated a lack of correlation between cellular or colonial variants of C. diphtheriae and fermentation reactions, it seems that in this instance the foregoing morphological and colonial changes accompanying the development of formaldehyde resistance also produced a variation from the control fermentation pattern.

The other biochemical changes induced in the resistant form were changes in the reduction of tellurite and in hemolytic capacity. Colonies grown in special media containing tellurite were the same rough (R) types noted above, having a gray color in contrast to the smooth (S), shiny-black colonies of the control. These same rough colonies appeared in cultures of the resistant forms on blood agar and did not produce hemolysis. Both of these changes are consistent with the

rough colony forms of this species (Morton, 1940) and are products of this feature rather than a peculiar aspect of formaldehyde resistance. The chromogenic property of the resistant colonies was unchanged.

Virulence tests and Ramon flocculation tests conducted on cultures and filtrates from the control organisms and from various phases during the development of formaldehyde resistance showed no changes in either virulence or toxin production. Thus the morphological, colonial, and biochemical variations observed had no observable effect on the virulence or toxigenicity. These facts are also similar to the lack of correlation of virulence and a specific colony form of *C. diptheria* noted by others and to the fact that diphtheria bacilli as identified by colony forms, fermentation reactions, and pigmentation may or may not elaborate specific exotoxin. Moreover, differences in the rate or quantity of toxin formation are a characteristic of strains and not associated with particular colony forms (Morton, 1940).

SUMMARY

An approximately 600-fold increase in resistance to formaldehyde was produced in a virulent, toxigenic culture of *Corynebacterium diphtheriae*, which could be maintained for at least 35 passages in the absence of the chemical. Certain morphological, cultural, and biochemical variations from the control accompanied the appearance of this resistance. However, no differences were noted in the virulence or toxigenicity of the formaldehyde-resistant cultures.

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THE EFFECT OF ESTERIFICATION OF PROTEIN CARBOXYL GROUPS ON THE STAINING OF BACTERIAL CELLS

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The exact mechanism by which the ordinary acid and basic dyes stain bacterial protoplasm is yet to be explained. The leading theories have been staining (1) by chemical union with the protoplasm, (2) by nonspecific physical adsorption onto surfaces, (3) by adsorption onto specific chemical sites, and (4) by solution in protoplasmic constituents. If staining reactions as a whole are considered, each of these theories will explain some particular case of staining. This paper is restricted in its scope to the staining of bacterial protoplasm by common basic and acid dyes.

Perhaps the most widely accepted theory of bacterial staining was presented by Stearn and Stearn (1929, 1930), who showed that acid dyes stain bacterial cells best under acid conditions and basic dyes stain best under basic conditions. This was explained by the amphoteric nature of proteins; thus, acid dyes could react with amino groups, and basic dyes could react with carboxyl groups, depending on the particular state of ionization of the protein. The idea of basic dyes combining with carboxyl groups of proteins was excellent. However, proof of this reaction in the case of bacterial protoplasm was indirect and many were not convinced.

Recently Fraenkel-Conrat and Olcott (1945) presented a method for the specific esterification of the carboxyl groups in protein, without altering the amino, sulfhydryl, or phenolic groups present. This was accomplished by exposure to methyl (or other) alcohol in the presence of 0.05 N HCl acting as a catalyst. If the concept of Stearn and Stearn is correct, it should be possible by using this method, to tie up the carboxyl groups in bacterial protein, thus destroying protoplasmic affinity for basic dyes. Affinity for acid dyes should remain. This result would give direct proof of the role of carboxyl groups in the staining of bacterial protoplasm by basic dyes. The following paper is a report on the effect of methylation on the staining of bacterial cells.

METHODS

Four species of gram-positive and four species of gram-negative organisms were employed. The organisms used were *Escherichia coli, Pseudomonas pavonacea, Serratia marcescens, Proteus vulgaris, Micrococcus aureus, Bacillus subtilis, Saccharomyces cereviseae*, and *Sarcina lutea*. Slides were prepared from distilled water suspensions of 24-hour nutrient agar slant cultures, and fixed by heat.

Esterification was accomplished according to the method of Fraenkel-Conrat and Olcott (1945) by immersing the slides in methyl alcohol containing 0.05 N HCl

as a catalyst. Control slides were treated with 0.05 N HCl in water, and another group of control slides received no treatment. At the end of 7 days the slides were removed, rinsed in distilled water, dried, and stained.

TABLE 1

Intensity of staining by basic dyes after one week of methylation

STAIN	pH	SLIDE	ORGANISM							
			E. coli	P. pavon-acea	S. mar-cescens	P. vul-garis	M. aureus	S. cere-visiae	B. sub-tilis	S. lutea
Safra-nine	11.5	C	++++	++++	++++	++++	++++	++++	++++	+++++
		M	+	+	+	±	++	++	++	++
Safra-nine	9.0	C	++++	++++	++++	++++	++++	++++	++++	+++++
		M	+	±	−	−	++	−	+	−
Methyl-ene blue	11.5	C	++++	++++	++++	++++	++++	++++	++++	++++
		M	+	−	±	−	+	−	+	−
Methyl-ene blue	9.0	C	++++	++++	+++	+++	++++	++++	++++	++++
		M	−	−	−	−	±	−	+	−

C, control slides treated with 0.05 N HCl alone. M, methylated slides.
Staining intensity is indicated by plus signs, lack of staining by a minus sign.

TABLE 2

Intensity of staining by acid fuchsin after one week of methylation

pH	SLIDE	ORGANISM							
		E. coli	P. pavon-acea	S. mar-cescens	P. vul-garis	M. aureus	B sub-tilis	S. cere-visiae	S. lutea
2.2	C	++	+	+	±	+	+	+	++
	M	++	+	+	+	++	++	++	++
5.0	C	+	+	+	+	±	±	+	+
	M	+++	++	++	++	++	++	+++	+++
7.0	C	−	−	−	−	−	−	−	−
	M	+	+	−	−	+	+	+	+

C, control slides treated with 0.05 N HCl alone. M, methylated slides.
Staining intensity is indicated by plus signs, lack of staining by a minus sign.

Two basic dyes, safranine and methylene blue, were employed as well as an acid dye, acid fuchsin. The safranine and methylene blue were dissolved in 0.5 per cent concentrations in buffered distilled water at pH 9.0 and 11.5. The acid fuchsin was prepared in the same concentration at pH 2.2, 5.0, and 7.0. Buffers

were made as described by Fraenkel-Conrat and Cooper (1944). A staining time of 1 minute was used for the basic dyes, and 10 minutes for the acid dye.

In the estimation of the staining intensity, methylated slides were visually compared with untreated control slides and the degree of staining was recorded by plus signs.

<div align="center">RESULTS</div>

Table 1 shows the results obtained when slides of the test organisms were methylated for 1 week at room temperature, followed by staining with safranine and methylene blue at pH 9.0 and 11.5. In every instance methylation resulted in either a complete loss of affinity for the basic dye or in a decided reduction in affinity. These results were obtained even though the basic dyes were used under conditions very favorable for their action.

Both groups of control slides stained with normal intensity. No difference was observed between the untreated controls and those exposed to 0.05 N aqueous HCl for 1 week.

Table 2 shows that methylated slides stained better with acid fuchsin than the untreated controls. This indicated that the amino groups were still present and able to receive the acid dye. The results also show that the amino groups were more available to the dye after the carboxyl groups had been esterified, since it can be seen that the treated cells stained faintly with the acid dye at pH 7, whereas the untreated cells did not.

<div align="center">DISCUSSION</div>

The results indicate that free carboxyl groups of bacterial protoplasm are involved in the staining reaction when a basic dye is used. This agrees with and gives direct proof of the concepts of Stearn and Stearn (1929, 1930). In view of the experiments presented here, it would appear that if the staining of protein by basic dyes is due to adsoption, it is adsorption at a specific chemical site. If this is admitted, then the difference between the chemical and adsorption theories is insignificant and staining could be explained by such terms as "chemical adsorption" or " adsorption exchange." Stearn and Stearn (1929) have pointed out that adsorption exchange follows the laws of ordinary chemical reactions, and that differentiation between chemical reactions and adsorption exchange becomes impossible as far as the staining reactions of bacterial cells are concerned.

Klotz and associates (1946, 1947) have made chemical investigations of complex formation between proteins and acid dyes and have concluded from their experiments that the reaction between the dye and protein involves the terminal amino group of lysine. This is analogous to the reaction between basic dyes and carboxyl groups as given in the present work. In some cases the methylated cells were reported as staining weakly with basic dyes. This result might have been due to residual free carboxyl or other acidic groups or to a purely nonspecific physical adsorption of the dye. If the latter were true, then obviously this process plays only a very minor role in staining.

The results presented in this paper apply primarily to the staining of bacteria by the ordinary basic dyes. There are some instances in which the staining mechanism is obviously different, such as the staining of fat by Sudan B. This staining can best be explained by the solubility theory as presented by Holmes (1926) and Conn and Holmes (1928).

SUMMARY

Esterification of the carboxyl groups of bacterial protein resulted in a loss of protoplasmic affinity for basic dyes; however, esterified cells continued to stain with acid fuchsin. The results indicate that basic dyes stain bacterial protoplasm by combination with free carboxyl groups.

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THE PREDOMINANCE OF COUNTERCLOCKWISE ROTATION DURING SWARMING OF BACILLUS SPECIES

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The tendency for growth or motion to follow a spiral path is very widespread in nature. Among the bacteria this is rather dramatically illustrated in the group movements exhibited by certain species that swarm over the surface of solid media. Because movement is confined to two dimensions in swarming, the phenomenon provides conditions that are suitable for a study of motion.

The swarming of species of *Proteus* has been observed in detail many times. Less well known are the peculiarities of swarming shown by some species of *Clostridium* (Turner and Eales, 1941b) and certain species of *Bacillus* (Muto, 1904; Henricksen and Svendsen, 1946; Roberts, 1935; Roszdestvensky and Medvinska, 1938; Russ-Münzer, 1938), notably *Bacillus circulans* (Smith and Clark, 1938; Turner and Eales, 1941a) and *Bacillus alvei* (Shinn, 1938; Smith and Clark, 1938; Shinn, 1940). Swarming among the *Eubacteriales* follows a general pattern (Murray and Elder, 1948). The extension over the agar surface is accomplished by groups of bacteria, moving as units. These units may take two forms: (1) "bullet-shaped" colonies of organisms moving at relatively high speed, almost always in curves, and (2) round colonies, rotating as plates on the surface of the medium, which may at the same time move in a direction. The bullet colonies seem to be common to all swarming species, but the rotating colonies are more highly developed in the swarming species of *Bacillus*, particularly *B. circulans*. The bullet colonies may travel in decreasing spirals until head meets tail and they become rotating colonies. Each type of colony leaves a trail of organisms from which originate new crops of swarming units.

In 1904 Muto described the general characteristics of swarming for an organism he named *Bacillus helixoides* (an organism that cannot be identified now although its general swarming characteristics resemble *B. circulans*). He noted that bullet colonies may become rotating colonies and that rotating colonies, originating from the track of another, do not necessarily rotate in the same direction as the "mother" colony. Other workers (Roberts, 1935; Russ-Münzer, 1938) have made similar observations since then. Russ-Münzer (1938) states: "The direction in which the colonies turn seems to be neither endogenous nor determined by the solidity of the agar—but to be determined by chance." Using *B. alvei*, Shinn in 1938 observed (using time-lapse cinephotography) that "of the probably 200–300 rotating colonies shown in the film, only two have been detected whose motion is clockwise." In 1940 Shinn made the statement that 95 per cent of the rotating colonies of *B. alvei* turned in a counterclockwise direction. Such a distribution would not be expected to occur by chance. Preliminary ob-

servations on this phenomenon have been reported in abstract by Murray and Elder (1949). In the present paper figures are presented which may be evaluated statistically.

METHODS

The swarming species of *Bacillus* used in this study were *B. circulans*, *B. alvei*, and *B. sphaericus* var. *rotans*. They were maintained and observed upon a papain broth medium (Asheshov, 1941) containing 1 per cent peptone (Difco) and 1.2 per cent agar (granulated, obtained from Agar Products, Ltd.). When fluid medium was used it consisted of the same medium without the agar. The organisms were grown and observed at room temperature.

Many of the observations were made by direct microscopy with a low-power objective (10 X) on the surface of agar plates. For some observations a modified form of "hanging block" preparation was used. For these preparations a thin layer of agar was flooded onto the surface of a cover glass, allowed to solidify, inoculated at a central point, and then inverted over a cell on a slide to which it was sealed. These preparations were used for observation with a 4-mm objective (45 X).

Directions of rotation or curvature are all expressed as if the observer were looking directly at the agar surface from above with the unaided eye.

OBSERVATIONS

The polarity of individual cells and swarming units. Observation of the vegetative cells of *B. circulans*, *B. alvei*, and *B. sphaericus* var. *rotans* in a fluid medium gave no hint of a constant polarity. When single cells were followed, some were observed to stop and frequently to restart in the reverse direction, without turning end for end. We must conclude, without any statistical evidence, that the individual cell has no constant polarity.

In swarming we are no longer dealing with the individual cell. The cells band together in groups, and each group has a polarity that is maintained until the group is disrupted by impact with a large mass or grows to a size restricting movement to zero. The principles that govern this aggregation into a swarming unit with polarity are unknown.

The relationship of swarming to motility. Those species among the *Eubacteriales* that exhibit swarming are all motile in fluid media and possess peritrichate flagella. The converse is not true, however, indicating some additional requirement for the ability to swarm. There is evidence (Boltjes, 1948) that swarming organisms have an extraordinarily large number of long flagella. This suggests that, in part, swarming may be a matter of motive power. Nonmotile variants occur (Clark, 1939) which have lost the ability to swarm.

With a technique suggested by Ørskov (1947), a thin layer of centrifuged India ink was placed in the path of swarming units and observed microscopically. Around the periphery of the moving unit it was seen that the particles of India Ink were agitated in a fashion suggesting flagellar activity.

If there is a direct relation between swarming and motility, substances that

interfere with the swarming phenomenon should act on motility in fluid media. With Congo red and dahlia violet (previously used to prevent swarming), bromthymol blue (accidentally found to inhibit swarming), sorbitan monooleate and "tergitol 4" (both surface-active agents) it was found that motility in fluid medium and swarming on solid medium were inhibited at the same concentration levels in each case.

We may conclude, therefore, that the motive power for swarming is provided by the same mechanism that propels individual organisms in fluid media; this mechanism is probably provided by the flagella, despite the contentions of Pijper (1946), which have been ably rebutted by Boltjes (1948) and others.

The direction of movement of swarming units. If it is assumed that the movement of individual cells in a fluid is at random, it would be expected that the movement of swarming units in their two-dimensional field also would be at random. On casual inspection this appears to be true, because a plate inoculated at a point becomes covered in a random fashion by the swarm. However, as has been stated, Shinn (1940) observed that the majority of rotating colonies of *B. alvei* revolve

TABLE 1

Direction of rotation of colonies

	CLOCKWISE		COUNTERCLOCKWISE		S.E.%
	Number observed	%	Number observed	%	
B. circulans 715	278	33.3	559	66.7	±1.6
B. alvei 662	71	32.0	156	68.0	±3.1
B. sphaericus var. *rotans* 633	57	34.5	108	65.5	±3.7

in one direction. To check this observation counts were made of the rotating colonies of *B. circulans*, *B. alvei*, and *B. sphaericus* var. *rotans*. The greatest proportion of colonies rotated in a counterclockwise direction, in a ratio close to 2:1 (table 1). There is no significant difference between the percentages for each species. It can be seen that the deviation from the expected 1:1 ratio is greater than would be expected by chance.

The bullet type of colony usually takes a curved path in migration across the agar. These curves might be expected to correspond with rotating colonies, especially in the case of *B. alvei* and *B. sphaericus* var. *rotans*, because a large proportion of the rotating colonies are derived from bullet colonies. The curves taken by all the bullet colonies observed were recorded for the instant of observation. The figures obtained are shown in table 2. The deflection of bullet colonies was predominantly counterclockwise, and the proportions observed were not significantly different from the figures for the rotating colonies.

One strain of each species was observed for the data so far presented. To check the constancy within a species, the deflections of bullet colonies in seven strains of *B. alvei* were recorded (table 3). There is good correspondence between the figures for each strain, and the predominance of counterclockwise deflection is main-

tained. We do not possess enough authenticated strains of the other species to do a similar study, although three strains of B. circulans behaved in the same fashion.

By careful subculture of individual moving units, attempts were made to isolate strains showing pure clockwise or counterclockwise deflection. All such attempts failed, confirming the experience of Russ-Münzer (1938). The resulting isolates all showed the same counterclockwise-clockwise ratio of 2:1 as did the parent culture.

Direction of movement according to colony size. This was investigated in the case of rotating colonies of B. circulans and not further pursued. No significant

TABLE 2

Direction of deflection of "bullet" colonies

	CLOCKWISE		COUNTERCLOCKWISE		S E.%
	Number observed	%	Number observed	%	
B. circulans 715	43	35.5	78	64.5	±4.35
B. alvei 662...	50	32.5	104	68.5	±3.8
B. sphaericus var. rotans 663	55	34.6	104	65.4	±3.8

TABLE 3

Comparison of different strains of B. alvei: direction of deflection of "bullet" colonies

STRAIN	CLOCKWISE		COUNTERCLOCKWISE	
	Number observed	%	Number observed	%
662	50	32.5	104	68.5
127	55	34.3	105	65.7
179	52	33.3	104	66.7
408	61	37.9	100	62.1
343	52	32.5	108	67.5
551	59	34.1	114	65.9
552	56	34.3	107	65.7

differences were detected in the ratios of clockwise-counterclockwise rotation in the size groups 0.025 to 0.075 mm and 0.075 to 0.15 mm. They are compared in a contingency table (table 4) in which the two groups show virtually identical distributions. It may be concluded that colony size has no effect, on the predominance of counterclockwise rotation.

The effect of position of the agar. It seemed possible that these movements might be affected by general external forces such as the rotation of the earth. Trials were made by maintaining the agar, after inoculation, in various positions relative to the earth's surface. No differences in the degree or characteristics of swarming were noted in any position. That the rotational field of the earth plays no part was shown by growing the organisms on duplicate plates, one oriented with the agar surface downwards and the other with the agar surface

upwards. It is shown in table 5 that the direction taken by the rotating colonies of *B. circulans* is in relation to the agar surface and not to the surface of the earth.

A comparison with B. cereus var. *mycoides.* The colonies of *B. cereus* var. *mycoides* are rhizoid and spreading. At the margin of these colonies can be seen projecting filaments that may curve in either a clockwise or counterclockwise direction. The mechanism of spreading is probably different in this case from that described for swarming. The organisms are either weakly motile or non-

TABLE 4

Contingency table comparing two size groups of rotating colonies of B. circulans

RANGE OF DIAMETER OF COLONY IN MM	NUMBER OF COLONIES	
	Clockwise	Counterclockwise
0.025–0.075	42 (41.3)	92 (92.2)
0.075–0.15	51 (51.2)	113 (112.8)

The expected values are in parentheses.
$\chi^2 = 0.00256.$ $p = 0.96.$

TABLE 5

The effect of position of the agar on colony rotation

B. CIRCULANS 715	DIRECTION OF ROTATION			
	Clockwise		Counterclockwise	
	Number observed	%	Number observed	%
Grown agar surface upward .	278	33.3	559	66.7
Grown agar surface downward	469	34.2	903	65.8

Directions are recorded as if looking at agar surface.

motile in fluid media, and on solid media the extension seems to be due to elongation by growth of the very long, rigid filaments that are characteristic of the organism. Although the mechanisms may not be analogous, the filamentous extensions are curved and can be enumerated. Fourteen strains of *B. cereus* var. *mycoides* were examined, of which eight produced predominantly counterclockwise curves. The results of counts upon these strains are shown in table 6. It can be seen that the predominantly counterclockwise strains are remarkably constant and on average are very close to a 3:1 ratio. The predominantly clockwise strains show a little more variability and the mean ratio lies between 2:1 and 3:1.

The phenomenon of elasticotaxis. This phenomenon has been described many times for *B. cereus* var. *mycoides* and was recently described for myxobacteria by

Stanier (1942) together with a neat method of demonstration. If a stress is put on
the agar, the filaments of B. cereus var. mycoides extend along the lines of stress
instead of following their usual curved path. Rectangular pieces of agar were cut
out of an agar plate and draped over a glass rod in a Petri dish. Inoculation
was made at a central point directly over the glass rod. The lines of stress run
straight down from the glass rod to the point at which the sheet of agar touches
the dish. The developing swarm was observed at frequent intervals. In the case
of B. alvei and B. sphaericus var. rotans, which produce predominantly bullet
colonies, there was good evidence, from the asymmetry of extension, that the
swarming units tended to follow the lines of stress. In the case of B. circulans,
which produces predominantly rotating colonies, the swarm extended in an even
and regular fashion giving no definite evidence of elasticotaxis. In this case,
although no counts were made, it was observed that both clockwise and counter-

TABLE 6

Curvature of terminal filaments of B. cereus var. mycoides

STRAIN	% CLOCKWISE	% COUNTER-CLOCKWISE	STRAIN	% CLOCKWISE	% COUNTER-CLOCKWISE
273	29	71	326	74	26
371	26	74	A1000	66	34
911	24	76	317	72	28
912	22	78	A967	73	27
936	23	77	A987	72	28
233	26	74	319	71	29
Mean.......	25	75	A966	64	36
			A306	62	38
			Mean ...	69.3	30.7

The total number observed for each strain was between 150 and 175.

clockwise rotating colonies were formed. The symmetry of swarm extension is
not affected by gravity.

The degree of elasticotaxis shown by bullet colonies may be due to the struc-
ture of the colony. Except at the anterior end, most of the bacilli are arranged
with their long axes in the direction of movement. The rotating colony is round
and the long axes of the bacilli are oriented tangentially.

Since elasticotaxis may be due to orientation of the long polymeric molecules
in the agar, attempt was made to get orientation by a different method. Agar
was poured on a slightly tilted, cooled plate of glass so that solidification occurred
during the flow. The method was extremely rough, but, in two out of ten trials,
preparations were obtained on which B. cereus var. mycoides showed definite
"elasticotaxis." With this result in mind, it was suspected that the time-honored
rotatory movement given to plates after pouring might be affecting the movement
of swarming units. However, comparison of counts made on plates that had been
swirled in either direction, or not at all, showed that this made no change in the
deflections observed for the bullet colonies of B. alvei.

DISCUSSION

From the morphological point of view there is no reason to expect polarity of the individual bacilli. The swarming species are all peritrichate and have no apparent constant asymmetry except a tendency to form excentric spores. We assume, for the time being, that individual cells swimming in a fluid have an unstable polarity that is constantly undergoing redistribution, possibly at random. In the swarming phenomenon we have aggregations of cells maintaining a remarkably stable polarity as a group, expressed as movement. From both direct and circumstantial evidence we conclude that swarming is an expression of motility under special conditions—in the thin fluid layer overlying the gel. Although the mechanism of aggregation into groups and the principle conferring polarity on that group are unknown, it would be expected that the units so formed would move at random in the two dimensions available to them.

Although a swarm covers an agar surface in a regular fashion from a point of origin, the evidence presented shows that the swarming units have an unexpected deviation from movement at random. This is expressed as a predominance of counterclockwise movement of rotating colonies and a predominance of counterclockwise curves taken by bullet colonies. Despite this deviation from the expected, swarms cover the surface of agar in a regular fashion because the migrating unit can start from the point of origin in any direction. The correspondence between rotating and bullet colonies might be expected since in many species the bullet colonies often take a tightening spiral path and become rotating colonies. In *B. circulans*, however, many rotating colonies are initiated by rotation in a small mass of actively growing cells without the intermediation of a bullet type of colony.

Since the position of the agar surface relative to gravity does not alter the phenomenon, the evidence is against, but does not exclude, the cause of this phenomenon being an external influence. From a consideration of elasticotaxis it is possible that the physical state of the agar conditions the effect. Although the bullet type of colony may be directed to some extent along lines of stress in the agar, the rotating colonies are not obviously affected. If the curvature of filaments of *B. cereus* var. *mycoides* is analogous to the rotation of other *Bacillus* species, then the development of both clockwise and counterclockwise strains on a single agar plate would be contrary to such a hypothesis.

There remains the possibility that these peculiarities of action are due to some inherent characteristic of individuals in the bacterial population. There is some basis for this hypothesis in the case of *B. cereus* var. *mycoides*. Gause (1939) suggested that the dextral form is a mutant of the more commonly occurring sinistral form in which the inversion of the growth of filaments is associated with the presence of D-isomers in the protoplasm. He supported his hypothesis to some extent by detecting in the dextral form an enzyme splitting the unnatural D-peptides (Gause, 1942). Alpatov and Nastyukova (1947) determined the relative toxicity of the optical isomers of mepacrine upon dextral and sinistral strains of *B. cereus* var. *mycoides*. The dextral form is inhibited to a greater extent by D-mepacrine, and the sinistral form is inhibited to a greater extent by

L-mepacrine. It must be emphasized that it is dangerous to compare too closely the activities of *B. cereus* var. *mycoides* and the swarming species of *Bacillus*. This is not only because the mechanisms of extension may be different but also because swarming strains have not been found that produce predominantly clockwise (dextral) deflections. However, the association is close enough—being related within the same genus, and the deflections being of the same general order—to warrant further study on biochemical lines and search for strains of *B. circulans*, *B. alvei*, and *B. sphaericus* that go contrary to the counterclockwise predominance demonstrated in this paper.

If the bacterial population is not homogeneous and consists of two variant types, of which one predominates slightly over the other determining the direction of deflection according to relative numbers, it would be possible to fit a hypothesis to the observed effect. In this case the ratios of deflection would have to vary, in the direction of the predominant type, in proportion to the number of individuals in the group. However, this hypothesis is upset by the close correspondence of the ratios observed for the rotating colonies of *B. circulans* grouped according to the size of colony.

It is not known whether such peculiarities may be detected in the motility of individual organisms. However, it could be suspected because individual filaments of *B. alvei* may be observed, on insufficiently dried agar, to move in a circular path like a toy train on a circular track. The fact remains that in swarming, which is a by-product of motility, the curved path traced by bullet colonies and the direction in which a colony rotates are not determined by chance.

ACKNOWLEDGMENTS

SUMMARY

The figures presented show that the swarming units of *Bacillus circulans*, *Bacillus alvei*, and *Bacillus sphaericus* var. *rotans* do not move at random. With each species the majority of rotating colonies turn in a counterclockwise direction in a ratio (counterclockwise:clockwise) of 2:1. The same ratio obtains when the curves taken by the migrating bullet-shaped colonies are enumerated. This ratio is remarkably constant and would not be expected by chance.

Evidence is given to show that the phenomenon is not likely to be due to external influences such as the rotational field of the earth or to a slight predominance of a variant in the bacterial population.

Comparison is made with the curving tendency of the terminal filaments around colonies of *Bacillus cereus* var. *mycoides*. In this species strains are found showing either predominantly clockwise or counterclockwise curves. Examination of a number of strains of each tendency shows that the ratios obtained are

close to 3:1 in all cases. The mechanism of colony extension is probably different from that of the other species. Strains of *B. circulans*, *B. alvei*, or *B. sphaericus* showing predominantly clockwise motion have not been found.

It is considered that swarming is an expression of motility in special restricted circumstances. If this is true it may be that the tendency to move in regular curves is an inherent property of organisms that is not detected in studies of motility.

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THE RELATIONS BETWEEN BOUND PENICILLIN AND GROWTH IN STAPHYLOCOCCUS AUREUS[1]

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Previous experiments (Maass and Johnson, 1949) have demonstrated that if a heavy suspension of resting cells of *Staphylococcus aureus* is treated with appropriate amounts of penicillin, approximately 0.8 units of penicillin are firmly bound to each ml of cells, exclusive of the penicillin penetrating the cells by simple diffusion. It was shown by the use of radioactive penicillin that this bound penicillin cannot be displaced by extensive washing of cells, nor by incubation with high concentrations of nonradioactive penicillin. The binding of penicillin by *S. aureus* cells has recently been confirmed by Cooper and Rowley (1949). These workers also observed, apparently consistently, reduced uptake by resistant *S. aureus* cultures, whereas in our experiments uptake less than half the normal was obtained in only 7 out of 15 trials.

A more direct approach to the problem of the antibiotic action of penicillin was made in the experiments reported below by further study of the relationship of the firmly bound penicillin to the growth and metabolism of the cells. These experiments were based on the hypothesis that penicillin forms a very firm complex with some essential component of the cell. The use of radioactive penicillin made it possible to study this complex from three different points of view. First, penicillin uptake at subbacteriostatic concentrations should differ from that at higher concentrations of penicillin, if the basic essential component theory is correct. Second, it might be expected that, if the cell were actively growing during exposure to penicillin, more penicillin might be taken up because of resynthesis of the blocked essential component. Third, it is possible that resting cells, which have been exposed to penicillin and have absorbed 0.8 units per ml, when transferred to a medium suitable for growth might displace some or all of the bound penicillin from its complex before growth occurs.

METHODS

Detailed procedures for growing cells for these experiments and the techniques used for the radioactivity determinations have been described (Maass and Johnson, 1949). Cells of *S. aureus* were grown in a rich aerated medium and harvested by a Sharples centrifuge. The culture used in the following experiments was a stock culture of *S. aureus* 209P sensitive to 0.05 units per ml of penicillin. Radioactive penicillin G was obtained biosynthetically from a fer-

mentation in a medium containing carrier-free S^{35}. The penicillin at the time of harvesting had a specific activity of 8,700 observed counts per minute per unit of penicillin. Cell suspensions or solutions tested for radioactivity were oxidized by fusion with sodium peroxide, and the sulfur was precipitated as $BaS^{35}O_4$ on flat plastic disks suitable for counting. The usual corrections for resolution time of the counter tube, for background, and for self-absorption were made. A medium containing 0.6 per cent peptone, 0.3 per cent yeast extract, 0.3 per cent beef extract, and 0.2 per cent glucose was used for growth experiments. The standard cup plate method with *S. aureus* H as the test organism was used for penicillin assays.

It was necessary in certain of these experiments to follow the increase in cell substance during growth or its constancy over long periods of incubation. The method used routinely was cell volume, measured in graduated centrifuge tubes or, when only small volumes were available, in hematocrit tubes. In either case, the suspensions were centrifuged until the observed cell volume was constant. At various times, cell weight, cell nitrogen, and turbidity were also determined. The density of dilute cell suspensions was followed by turbidity measurements in the Evelyn colorimeter using a 660-mμ filter. Dry weights were measured by weighing a dried sample of washed cells in a tared centrifuge tube. Cell nitrogen was determined on washed cells by the micro method of Johnson (1941). These methods all yielded similar data. A typical comparison is given in figure 2.

RESULTS

Uptake of penicillin at subbacteriostatic penicillin concentrations. Four-hundred-ml portions of a cell suspension containing 8 ml of cells (2 per cent by volume) were incubated in 2-liter Erlenmeyer flasks on a reciprocating shaker at 30 C with radioactive penicillin at two concentrations—0.01 unit per ml and 0.04 units per ml. These suspensions were sampled at various times, and the penicillin bound by the cells was determined. Representative data are shown in figure 1. It may be seen that, at these low concentrations, binding of penicillin was slow. This low reaction rate is not surprising in view of the fact that a penicillin solution containing 0.01 unit per ml is approximately 1.7×10^{-8} molar. It will be further noted that the rate of reaction is approximately proportional to the penicillin concentration. This is brought out in figure 1, in which the points are experimental, but the two solid curves are drawn so that, for any ordinate, the abscissa of one is four times the abscissa of the other.

This experiment was based on the assumption that the volume of cells remained relatively constant during the long period of incubation. The data of table 1, giving results of three methods of determining cell substance, indicate that this assumption was justified.

Uptake of penicillin by growing cells. Five-hundred-ml portions of medium in 2-liter Erlenmeyer flasks were inoculated with 5 ml of a 24-hour culture of *S. aureus* 209P. The cultures were incubated at 30 C on a reciprocating shaker until turbidity measurements showed that the cells were at the beginning stage of the logarithmic phase of growth. Four hours' incubation was sufficient for

this purpose. One portion of the culture was then centrifuged; the residual cells were suspended in a volume of pH 6.1 phosphate buffer equal to the volume of the cells, and treated with 0.4 units per ml of penicillin. This particular concentration was selected because it was definitely in the bacteriostatic range, and
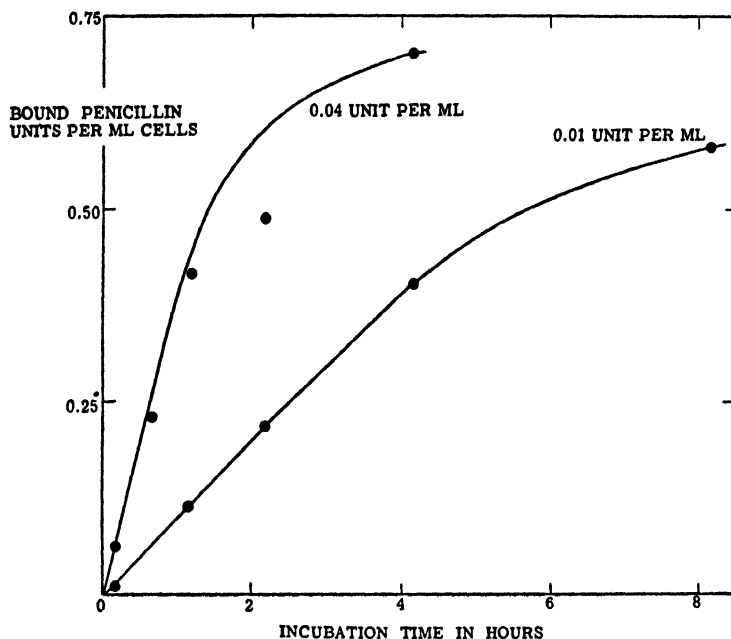
Figure 1. Penicillin uptake by cells of *S. aureus* at two subbacteriostatic concentrations of penicillin.

TABLE 1

Effect of time of incubation of cell suspensions on integrity of the cells

PENICILLIN CONCEN-TRATION IN MEDIUM	TIME OF INCUBATION	CELL SUBSTANCE INDICATED BY		
		Hematocrit values	Dry weight	Cell nitrogen
units/ml	*hours*	*% by volume*	*mg/ml*	*µg/ml*
0.01	0	2.0	5.85	2,250
	8	1.8	5.47	1,990
0.04	0	2.0	5.85	2,250
	4	1.6	5.85	2,120

yet low enough so that small amounts of medium contaminating the cells would not significantly affect the determination of penicillin in the cells. The penicillin bound by these cells was then determined. Data from this part of the experiments are shown in the first section of table 2. At the same time, 0.4 units per ml of penicillin were added to another portion of the culture and incubation on the shaker at 30 C was continued for 2 hours. The amount of penicillin taken up by these cells is shown in the second section of table 2. A third portion of the

culture was grown for a total of 6 hours and then treated with the same amount of penicillin. The mixture was centrifuged and the bound penicillin in the cells

TABLE 2

Penicillin uptake by growing cells

NO. OF EXPT.	CELLS GROWN 4 HR, THEN TREATED WITH PENICILLIN		CELLS GROWN 4 HR, THEN GROWN 2 HR WITH PENICILLIN		CELLS GROWN 6 HR, THEN TREATED WITH PENICILLIN	
	Penicillin bound	Cell volume*	Penicillin bound	Cell volume*	Penicillin bound	Cell volume*
	units/ml	*ml*	*units/ml*	*ml*	*units/ml*	*ml*
1	0.76	2.1	1.62	2.5	0.80	4.5
2	0.58	2.2	2.06	2.4	0.66	7.7
3	0.56	3.0	1.74	3.6	0.57	6.5

* Per 500 ml culture.
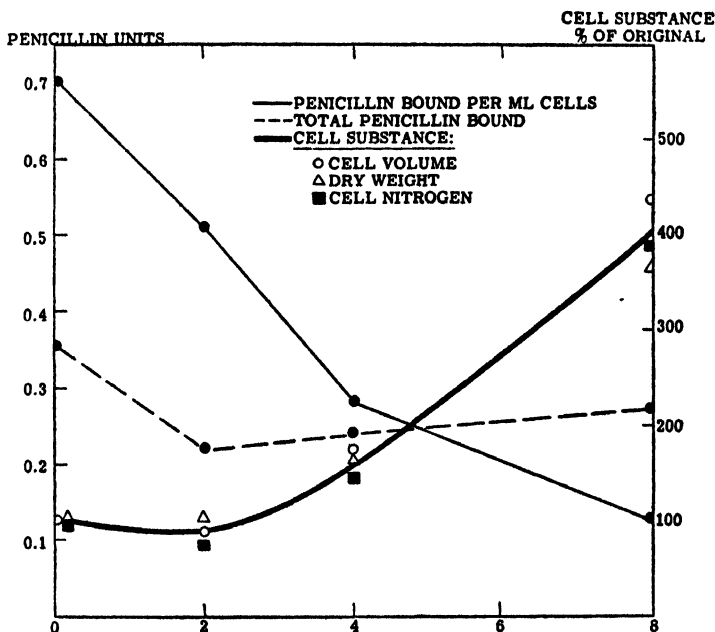
Figure 2. Displacement of bound penicillin on growth of cells in a fresh medium. Bound penicillin is plotted in terms of units per ml and also in total units. The curve for cell substance represents an average of three criteria—cell nitrogen, cell volume, and dry weight—expressed as percentage of the original value. Original values are as follows: cell nitrogen, 98 μg per ml; dry weight, 0.293 mg per ml; cell volume, 0.49 ml per 500 ml medium.

determined. These results are indicated in the third section of table 2. The total volume of cells produced in each case is also shown.

It may readily be seen from table 2 that cells growing in the presence of penicillin for 2 hours absorb two to three times more penicillin than cells grown under the same conditions and treated as resting cells with penicillin. As has previously been shown, 2-hour incubation of resting cells at penicillin concen-

trations of this order result in no greater uptake than 10-minute incubation. Cooper and Rowley (1949) have also recently reported that growing *S. aureus* cells bind more penicillin than resting cells.

Another observation that may be made from the data of table 2 is that the amount of penicillin bound by nongrowing cells is the same, whether they are grown for 4 hours and then treated with penicillin in heavy suspension or grown for 6 hours and mixed with penicillin for a short time in the original medium.

Displacement of bound penicillin by growth of penicillin-treated cells in fresh medium. Ten ml of packed cells were mixed with 10 ml of buffer containing 1 unit per ml of penicillin. After thorough washing of the cells, the penicillin absorbed by the cells was shown to be 0.70 units per ml of cells. One-half ml of these cells was then used to inoculate 500 ml of medium. The penicillin remaining on the cells was determined at 2, 4, and 8 hours. The growth of the cells was also followed during this time by the determination of cell volume in hematocrit tubes, by dry weight, and by cell nitrogen. The data of one experiment are shown in figure 2.

Consideration of the curve for penicillin bound expressed in units per ml would lead to the conclusion that there is a marked decrease in the amount of penicillin bound, and that a large displacement has occurred. However, if the total penicillin bound, regardless of cell volume, is plotted against time, it may be seen that only during the lag phase is there any decrease in the penicillin in the cells, and that this decrease is relatively slight. As the cells grow and increase in volume, the remaining penicillin is diluted by the division of the cells.

<center>DISCUSSION</center>

The data presented above and in the previous paper (Maass and Johnson, 1949) throw some light on the differences in penicillin action on resting, slowly growing, and rapidly growing cells. There is a considerable body of evidence in support of the hypothesis that penicillin inhibits some reaction essential to cell division, but not essential to respiration or to synthesis of many cell components (Chain and Duthie, 1945; Gale, 1948; Parker and Luse, 1948). The demonstration of extremely firm binding of a very small and relatively constant amount of penicillin by sensitive cells is an indication that some cell component, present in small amounts, combines irreversibly and rapidly with penicillin. That all of this component in the cell is combined with penicillin is indicated by the fact that the amount of combined penicillin is independent, within wide limits, of penicillin concentration and time of incubation with penicillin. If the obvious conclusion is made that this penicillin combines with a cell component essential to a reaction involved in cell division, it appears likely that this component must be a catalyst rather than a metabolite, because of the extremely small quantities involved. (Approximately 2×10^{-9} moles of penicillin are fixed by 1 ml of cells.)

Parker and Luse (1948) have suggested that the cell growth occurring when penicillin-treated cells are transferred to penicillin-free medium might occur only after resynthesis of an exhausted metabolite. The present experiments sug-

gest that resynthesis of an adequate amount of penicillin-binding component may be the chief requisite for renewed growth. From table 2 it may be seen that cells in which all the penicillin-binding component has been blocked resynthesize the new component two to three times as rapidly as cell substance. In the absence of penicillin, this resynthesis should rapidly have restored the cell to normal, permitting growth. It is interesting to note (figure 2) that the bulk of the penicillin bound by cells is not excreted when renewed growth and multiplication occurs, but remains in the daughter cells.

In view of the fact that the penicillin-binding component appears to fix penicillin even at minute concentrations and that bound radioactive penicillin does not exchange with high concentrations of free penicillin, some factor other than a dissociation constant of the bound penicillin must be responsible for the multiplication of cells at penicillin levels below the bacteriostatic concentration. The strain of S. aureus used in these experiments will show slight growth at 0.05 units per ml of penicillin, but no growth at 0.06 units per ml. The lag phase after transfer of the organism is approximately 2 hours. It may be estimated from the data of figure 1 that at a penicillin concentration of 0.06 units per ml, blocking of the penicillin-binding component would be almost complete in 2 hours. It therefore appears reasonable that the lowest bacteriostatic penicillin concentration is the concentration at which blocking of the penicillin-binding component is slightly more rapid than its resynthesis by the cell.

SUMMARY

The penicillin previously reported to be firmly bound by resting *Staphylococcus aureus* cells has been found to remain largely bound during subsequent multiplication of the cells in a penicillin-free medium.

At low penicillin concentrations (0.01 to 0.04 units per ml) penicillin uptake is apparently unchanged in amount, but is much slower, many hours being required for completion of the reaction.

In a medium capable of supporting growth, cells in the presence of penicillin continue to bind penicillin, indicating more rapid synthesis of the penicillin-binding component than of cell substance.

The relation of these findings to the mode of action of penicillin is discussed.

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GELATINOUS VARIANTS OF PSEUDOMONAS AERUGINOSA[1]

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Pseudomonas aeruginosa is known to show considerable variation in colony form and physiological properties, including pigment production. Gaby (1946) has recently reported biochemical and other observations on certain "basic colony types." Mucoid variants of *P. aeruginosa* have been described by Sonnenschein (1927) and by Schwarz and Lazarus (1947); Fiala (1941) has described the appearance of "rugose" colonial forms when the organism was grown on glycerinated media. None of the colonial variations described so far, however, correspond to the gelatinous variants referred to in this paper.

In 1943 Schultz (1947) isolated a strain of *P. aeruginosa* from an infected third-degree burn which, when plated on maltose Sabouraud's medium supplemented with 0.5 per cent glycerol, produced a few unique colonies intermingled with normal colony forms. These unique types were smooth, dome-shaped, translucent colonies, of a stiff, jellylike consistency, and resembled small pearls. After a number of replatings some of these well-rounded "pearl forms" developed an irregular surface contour (rugose gelatinous forms). On glycerinated Sabouraud's medium (GSM) the colonies are as stiff as molded gelatin and can be lifted intact from the substrate. Gelatinous colonies continue to grow much longer than ordinary bacterial colonies and often become of extraordinary size, in 2 to 3 weeks frequently reaching over a centimeter in diameter and more than a half a centimeter in height. Colonies of certain gelatinous forms grow until they touch the lid of an inverted petri dish. In these, the colonies often taper from a centimeter in diameter at the base to 2 or 3 mm at the tip. Many, however, are more rounded or dome-shaped, and present surface irregularities of the kind one would expect were heavy rounded cords of the gelatinous material drawn through the colony. The gelatinous colonies initially observed were smooth and beadlike, but later platings contained more of the "rugose" gelatinous type; the latter, however, sometimes reverted to the smooth type.

Gelatinous colonies are not produced by gelatinous variants when seeded to ordinary nutrient agar. Certain ingredients, such as glycerol, seem to be necessary in a medium for their production. Moreover, not all gelatinous colony strains or "clones"[2] necessarily produce solely gelatinous colonies on replating, even though these are grown continually on a glycerol-containing substrate. On further plating, gelatinous clones nearly always produce a few nongelatinous

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colonies, even on a glycerol medium. In some instances, however, gelatinous clones appear to be exceedingly stable and show little tendency to revert to ordinary nongelatinous forms. The variation in question appears to be one of capacity to convert glycerol, and certain other substances, into the abundant gelatinous material chiefly responsible for the size and consistency of the colony.

The present study was undertaken (1) to determine whether chemical substances other than glycerol can be utilized by gelatinous variants for the production of gelatinous colonies, (2) to determine the nature of the gelatinous material produced, (3) to determine by "histological" methods the internal structure of these colonies, (4) to determine whether these variants are antigenically distinguishable from ordinary nongelatinous forms of *P. aeruginosa*, and (5) to determine to what extent, if any, ordinary laboratory strains of *P. aeruginosa* may show gelatinous variants when placed on appropriate media.

METHODS AND MATERIALS

In the investigation of the substrates on which the gelatinous variants produce their characteristic colonies, ordinary media such as nutrient agar, veal agar, blood agar, and Sabouraud's maltose agar were employed, with and without 0.5 to 1 per cent glycerol. In addition, media were employed which contained such alcohols as methyl, ethyl, propyl, butyl, amyl, ethylene glycol, erythritol, mannitol, and sorbitol. These alcohols were employed in concentrations of 1 to 3 per cent. Other media contained vegetable oil, added to Starr's medium as a base.

As a rule the individual colonies under study were streaked directly from one plate to another, without making intermediate broth cultures. In plating from glycerinated to nonglycerinated media, however, intermediate broth cultures were generally employed to avoid the possibility of carrying over small amounts of glycerol to the new solid medium. About 20 ml of medium were poured into each plate to provide adequate nutriment and moisture for good growth. After the plates were carefully streaked, they were inverted and placed under a bell jar together with a beaker of water to maintain sufficient moisture for the more or less long period of growth. With minor exceptions the plates were incubated at room temperature.

A procedure for the isolation of pneumococcus polysaccharide (Goebel, 1930) was employed in our attempt to determine the general chemical nature of the gelatinous material produced, the gelatinous material being repeatedly precipitated with 95 per cent ethyl alcohol and redissolved in water. Trichloracetic acid was employed to remove the protein present.

In the preparation of sections of the colonies, fixation was obtained by placing the colony and underlying block of agar, cut around the colony, in a 1:1 solution of 80 per cent alcohol and formalin, according to the method of Legroux and Magrou (1920) in fixing colonies of *Vibrio comma*. The colony was then dehydrated by passage through a series of alcohols, after which it was put through xylol and embedded in paraffin. Sections were then made with an ordinary tissue microtome, and these were fixed to slides and stained according to the procedure suggested by Legroux and Magrou or with dilute carbol fuchsin.

In the work on the antigenic properties of the gelatinous variant, antisera were prepared in rabbits. The latter were immunized with three kinds of antigens. Certain of the animals were immunized with clone 180a, a gelatinous clone grown on a glycerinated medium for the purpose of including the gelatinous substance of the colony in the vaccine; another group was immunized with the same clone grown on nutrient agar, on which no gelatinous material is produced; and still another group was immunized with clone 106, a nongelatinous clone of the same strain of *Pseudomonas* grown on nutrient agar. In all three instances the organisms were grown for 18 hours at 37 C. Suspensions of these cultures were prepared in 0.4 per cent formalin in physiological saline solution. After centrifugation the number of organisms was adjusted to nephelometer no. 5, i.e., 1,500,-000 organisms per ml, and the vaccines were held for 1 hour at 60 C in a water bath. The rabbits were injected intravenously three times a week with these preparations, and then allowed to rest for a week, until nine injections had been given. The doses ranged from 0.1 ml initially to 0.5 ml later, the total being 3.1 ml. Blood was collected by cardiac puncture 1 week after the last injection.

OBSERVATIONS

Types of gelatinous colonies. On glycerinated Sabouraud's medium at least three distinctive types of gelatinous colonies have been observed: (1) an evenly rounded, dome-shaped colony resembling a small pearl ("pearl form"), (2) a dome-shaped colony with an uneven, essentially mulberry type of surface ("mulberry form"), and (3) one with a centrally placed tonguelike projection ("tongue form"). As pointed out by Schultz (1947), these colony types correspond to certain differences that may be observed among ordinary nongelatinous colonies of *P. aeruginosa*. The pearl form has its counterpart in the smooth, convex, nongelatinous colony; the mulberry form has its counterpart in the rugose nongelatinous colony; and the tongue form has its counterpart in an umbonate type of nongelatinous colony. In other words, the ability to produce the gelatinous material is not restricted to any particular basic colony type. Moreover, as mentioned earlier, these types do not always remain true to form. Variations may occur among gelatinous variants similar to those that may occur among nongelatinous forms. The tongue form colonies nearly always give rise to some of the mulberry form; the pearl form colonies in general tend to become the mulberry type, corresponding to the tendency of smooth, nongelatinous colonies to become rugose, especially when grown on glycerinated media (Fiala, 1941). Sometimes colonies of the mulberry type give rise to pearl colonies or tongue forms. A few nongelatinous colonies nearly always arise from gelatinous colonies on repeated replating, colonies that remain nongelatinous even though repeatedly transferred to glycerinated media.

Media ingredients which determine the production of gelatinous material by gelatinous variants. In initiating the present study we repeated observations made earlier (Schultz, 1947) showing that glycerol was essential for the production of gelatinous colonies. Ordinary media with and without glycerol were tried; these routine media included Sabouraud's medium, nutrient agar, veal agar, blood agar, and Veillon's agar. Without the presence of glycerol none of these favored

the production of gelatinous material by the gelatinous variant. With glycerol present, gelatinous colonies were produced by the gelatinous variant in all instances, except on blood agar. Something in the blood agar clearly prevented the formation of the characteristic gelatinous material. Although gelatinous material was produced on the media mentioned, it should be noted that the gelatinous material produced on the different glycerinated media was not of the
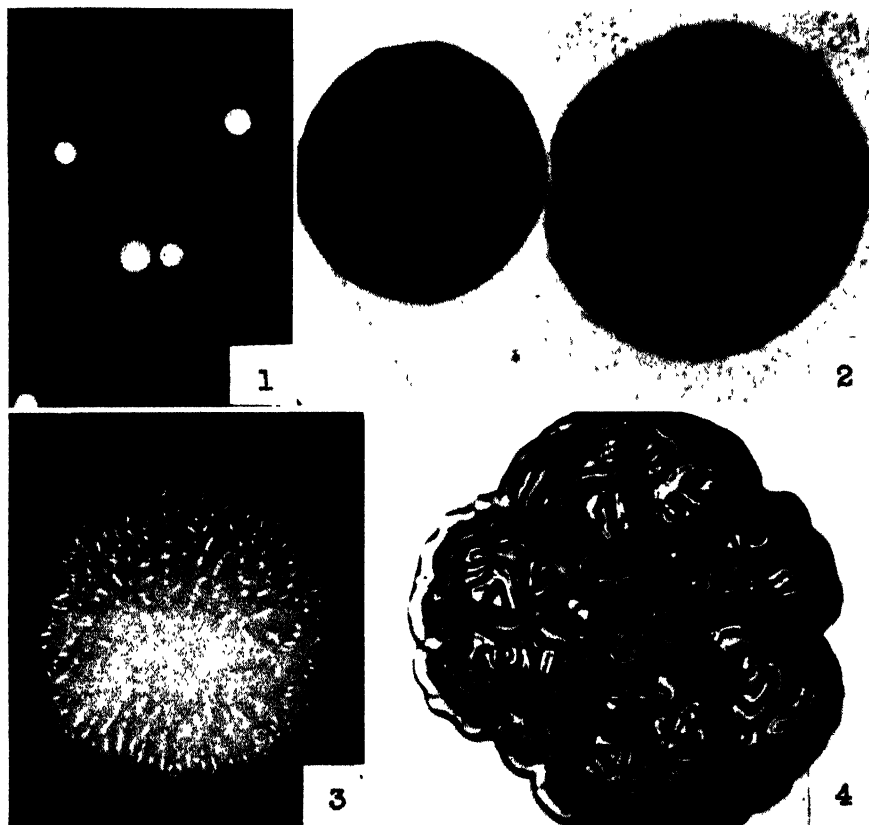
Figure 1. Small gelatinous colonies of the smooth type ("pearl forms") by reflected light.
Figure 2. An opaque smooth gelatinous colony (left) and a smooth nongelatinous colony, both by transmitted light.
Figure 3. Rugose gelatinous colony by reflected light.
Figure 4. Rugose nongelatinous colony by transmitted light.

same consistency. On glycerinated veal agar, for example, the gelatinous material is comparatively soft and drips readily onto the lid of the inverted petri dish, but on GSM and on glycerinated nutrient agar the material is stiff and the colonies assume well-defined shapes. Starch agar was tested to see whether a polysaccharide would prove a suitable substrate for the production of gelatinous colonies, but none were produced. Glucose agar also failed to yield them.

Several alcohols were tested. With the monohydroxy alcohols, employed in 3 per cent amounts, the following results were obtained: on methyl alcohol agar

very soft gelatinous colonies were produced and growth was restricted; on ethyl alcohol agar, growth was better and the colonies were more definitely gelatinous in character; on propyl alcohol agar, true gelatinous colonies were produced and a new type of gelatinous colony was observed when one of the clones was seeded to this medium. This clone, which usually produced a tall umbonate type of gelatinous colony on GSM, on propyl alcohol agar produced an almost saucer-shaped gelatinous type of colony. Although definitely gelatinous it failed to grow much in height. It was associated, however, with other colonies that did reach considerable height. On replating this flat variety, only the flat type was reproduced. It was in no sense rugose and had the appearance of a saucer with a well-defined rolled edge. This was the only observed instance of a more or less flat gelatinous colony.

On butyl alcohol agar stiff gelatinous colonies were produced. The organisms grew well on this medium although the colonies were somewhat smaller than those produced on GSM. On amyl alcohol agar the organisms required almost a week to show definite growth. Only a small amount of gelatinous material was produced and this was very dry. On second plating, growth occurred more rapidly and more gelatinous substance was produced. When 2 per cent of the dihydroxy alcohol, ethylene glycol, was incorporated in nutrient agar, the gelatinous variant produced small and very soft gelatinous colonies. The tetrahydroxy alcohol, erythritol, was employed in a 1 per cent amount. No true gelatinous colonies were produced, but instead there were round, butyrous colonies with slightly raised centers having a very thin jellylike consistency. The pentahydroxy alcohol, adonitol, was also employed in a 1 per cent amount. In this case no gelatinous colonies at all were formed, but instead there were round, butyrous colonies with slightly raised centers.

The hexahydroxy alcohols, mannitol and sorbitol, were each employed in 1 per cent amounts. On the sorbitol medium no gelatinous colonies were formed. On mannitol, an isomer of sorbitol, they were formed, however. The gelatinous material was relatively fluid and it was produced in such abundance that it dripped freely onto the lid of the inverted petri dish. On the other hand, some of the colonies were quite stiff with smooth surfaces and looked very much like pearls.

Since *P. aeruginosa* is one of the few bacterial species possessing a fat-hydrolyzing enzyme system, it was thought that the gelatinous variant might split fats and utilize the glycerol contained in them. The four fats tested were peanut oil, vegetable oil, cod-liver oil, and table butter. The organisms grew very well on all of these media, and typical gelatinous colonies were produced. The nongelatinous forms showed no such tendency. The best growth occurred on the medium containing butter. Here and there on a few of the plates areas of turquoise blue were observed, indicating that the fat had been hydrolyzed. These areas were relatively few, however.

The effect of aging cultures of the gelatinous variant. Gelatinous and nongelatinous forms of the organism were seeded to Martin's peptone solution with and without 1 per cent glycerol. These cultures were held at room temperature.

While the gelatinous variants in time transformed the medium containing the glycerol into a gelatinous, soft, jellylike material, which became more and more gelatinous on gradual dehydration, the nongelatinous forms failed to show this feature. In Martin's peptone, without glycerol, none of this jellylike material was formed by either the gelatinous or nongelatinous forms.

After these cultures had been allowed to stand for about 4 months, they were seeded to GSM. No new types of gelatinous colonies resulted from this "aging." It was observed, however, that while the peptone solution with glycerol favored the maintenance of the gelatinous variants, the solution without glycerol favored the appearance of the nongelatinous forms. This was made obvious by the fact that many more nongelatinous forms were intermingled with gelatinous colonies on GSM plates seeded from glycerol-free broth than was the case when the seedings were made from glycerol peptone solution. There was one exception to this observation. In one instance, a gelatinous variant transferred from glycerol peptone solution to GSM, only one small gelatinous colony was formed, all of the remaining colonies being of the nongelatinous type. In this case then the variant has reverted to the usual form shown by pyocyaneus strains, despite its growth in the presence of glycerol.

The effect of phage on colony variation. At the same time that the gelatinous variant was isolated, a strong pyocyaneus phage was isolated from the same source. It was thought that the presence of phage might have had something to do with the production of this variant. Schultz (1947), however, has reported that both the gelatinous and the nongelatinous variants were equally susceptible to the action of the phage isolated. When this work was repeated with three nongelatinous and five gelatinous forms, it was found that the three nongelatinous forms were highly susceptible to the phage, whereas only two of the five gelatinous variants were susceptible. The lytic activity was tested in broth cultures only.

Secondary cultures of phage-lysed clones were seeded to GSM. Gelatinous colonies were still formed by the secondary organism arising from the lysed gelatinous clones, and nongelatinous colonies from secondary cultures of nongelatinous clones. It would seem, therefore, that phage action had not been initially responsible for the production of the gelatinous variant, since no gelatinous colonies appeared on plates seeded from secondary cultures of the nongelatinous forms.

Morphological characteristics of individual cells. The individual cells of the gelatinous clone are morphologically indistinguishable from those of a nongelatinous clone. It was thought that perhaps the gelatinous material might be present in the form of a capsule. Attempts were made to demonstrate capsules on gelatinous variants in cultures of different ages, in both liquid and solid media either free from or enriched with glycerol. Only occasionally was what appeared to be a definite capsule observed. The "negative" staining methods employing Congo red and acid alcohol, Hiss's capsule stain, and Anthony's method using skimmed milk as a background were tried. The ability to produce capsules would therefore appear to be either quite variable or difficult to establish with certainty. .

Stock strains of P. aeruginosa tested for gelatinous variants. The stock strains of *P. aeruginosa* in this laboratory had been carried for several years on glycerol agar. These were examined from time to time for gelatinous variants by streaking them on GSM plates. The results were uniformly negative until recently, when a small gelatinous colony appeared on a slant of glycerinated agar streaked with one of these stock strains. On transferring this to GSM and to glycerinated agar, rugose gelatinous colonies with definite umbonate centers appeared. There were no butyrous colonies on the plates. However, when one of these colonies was transferred to glycerol-free broth and then back again to GSM, quite a number of butyrous colonies appeared along with small gelatinous colonies.

Antigenic properties. No antibodies were demonstrable by means of the agglutination test in the sera of the rabbits injected as described under "Methods." However, sera collected from the rabbits injected with clones 180a and 106 grown in glycerol-free media did fix complement in serum dilutions out to 1:512. A second series of injections was therefore given to the rabbits by intraperitoneal and subcutaneous routes. The organisms were grown on a glycerol-free solid medium, and, after centrifugation, physiological saline solution at 85 C was added to the organisms and the suspensions were then immersed in an 85 C water bath for 15 minutes.

Following the second series of injections, good agglutination was obtained but the agglutinin titer was low, the highest active dilution being 1:16 in both cases. Although these antisera were of low titer, they seemed to establish that the gelatinous clones do not constitute a distinct antigenic group. While differences were observed among the clones tested, there was no correlation between agglutinability and ability or lack of ability to produce gelatinous colonies.

Chemical nature of the gelatinous material. Gelatinous colonies were scraped from solid media and put into the following solvents to determine their solubility. The results were as follows:

Carbon tetrachloride	Insoluble
Ether	"
HCl, 0.2%	"
Alcohol, 70%	"
Alcohol, 50%	"
NaOH, conc.	Slightly soluble
NaCl, 10%	Almost completely soluble (slow)
Saline, 0.85%	" " " "
Water, at room temperature	" " " "
Water, boiling	Completely soluble
HCl, conc.	" "
Na$_2$CO$_3$, 0.5%	" "

Gelatinous colonies, from 10- day- to 2-week-old cultures, were scraped from the surface of a solid medium and put into hot water, which was then brought to a boiling temperature for 2 to 3 minutes to allow all of the gelatinous substance to go into solution. As this solution cooled, it took on a jellylike consis-

tency. When this was poured into 95 per cent ethyl alcohol, a large, amorphous, gelatinous mass precipitated out. This retained some of the pigment, but most of the pigment remained in solution. This precipitate was readily redissolved in hot water, from which it could be reprecipitated by alcohol. The material precipitated by alcohol consisted of strands which had slight elastic properties. These were not strong, however, and could be torn apart readily. On dehydration they became brittle, but still went back into solution in hot water. The solution now became more viscous than water but not jellylike.

When 10 ml of a saturated solution of trichloracetic acid was added to 100 ml of an aqueous solution of the alcohol precipitate of the gelatinous material, only a scant precipitate formed. However, when the filtrate was neutralized with sodium hydroxide and this was then added to 95 per cent alcohol, a considerable amount of amorphous material was then precipitated from the solution. This could be redissolved in hot water. When a 1:1 hydrochloric acid solution was added at 0 C, no precipitate was formed—such as is obtained in purifying pneumococcus polysaccharide (Goebel, 1930). The material remained fully in solution.

Millon's test for protein was at no time positive on this gelatinous material, not even in the first aqueous solution of gelatinous colonies. The Molisch test for carbohydrate was strongly positive in the first aqueous solution of the gelatinous material but became less so with each step of the purification procedure.

The initial aqueous solution of the gelatinous material did not reduce Benedict's solution, nor did this test become positive after this solution was boiled with hydrochloric acid. The iodine test for starch also proved negative throughout.

It is difficult to draw any conclusions from these results other than the following: that the gelatinous material produced in this type of colony appears to be carbohydrate of some sort but that its exact nature remains to be determined by further investigation.

Since antibiotic activities are exhibited by *P. aeruginosa*, it was decided also to test this gelatinous material for antibiotic properties. The cup plate method was employed, and the partially purified gelatinous material was tested against laboratory strains of *Staphylococcus aureus* and *Escherichia coli*. No antibiotic activity was observed.

Observations on colony sections. In the preparation of paraffin sections of the gelatinous colonies two difficulties were encountered in fixing the sections to the slide and in getting them well stained. Colonies grown on glycerol agar proved much too soluble in water to permit contact with water even for a few seconds. When sections were brought in contact with water they began to swell and disintegrate almost immediately. Colonies grown on GSM or butyl alcohol agar proved a little easier to handle, and a few could be fixed to a slide without noticeable distortion. To accomplish this required rapid work with water barely warm enough to flatten the paraffin ribbon.

The staining method employed by Legroux and Magrou (1920) in their work with colonies of *Vibrio comma* proved fairly effective in staining colony sections

of the gelatinous variants here being reported. By this method the organisms in the colony stained blue and the gelatinous matrix pink. When stained with dilute carbol fuchsin the matrix stained a light red and the organisms merely a deeper red.

A marked difference was observed in the consistency of the colony, and in the distribution of the organisms, in young and old colonies. Colonies of about 5 days of age consisted almost entirely of organisms with little in the way of a gelatinous matrix. Colonies 10 days to 2 months of age, on the other hand, showed a large amount of matrix. This consisted largely of more or less heavy strands, which often imparted an alveolar type of structure to much of the colony. The distribution of the organisms in these colonies differed. In some the organisms seemed to be more or less evenly distributed, in others only clumps or small collections of cells could be identified here and there throughout the colony. Technical reasons may have been partially responsible for these differences. The staining procedure, for example, may not have been adequate to show up all the organisms present, or some of the organisms may have been washed away in fixing the sections to the slides.

It was thought that perhaps the sections would show more clearly whether capsules are produced by these variants, something which could not be definitely established by the other methods (see above). However, in only one colony were there appearances that suggested capsule formation.

DISCUSSION

Since the manifestation of such a variation as is here described depends upon the presence of certain ingredients in the substrate, it is obvious that it can be easily missed unless an appropriate medium happens to be employed. In this case the presence of glycerol brought the variant to light, the initial isolation of the variant having been obtained when the exudate from a pyocyaneus-infected lesion was seeded directly to Sabouraud's medium supplemented with glycerol, a medium that was employed to rule out a possible concomitant fungus infection. Glycerinated media are ordinarily not employed in the cultivaion of *P. aeruginosa* from lesions. It is of interest that only a very few gelatinous colonies appeared among the numerous ordinary or nongelatinous colonies of *P. aeruginosa* that grew on the original plates. This suggests that relatively few of the organisms originally seeded possessed the power to produce this thick gelatinous material in the presence of glycerol. Those originally present were all of the smooth, evenly rounded pearl forms, but in subsequent transplantation many of these gradually transformed into gelatinous forms that differed from the original pearl forms (Schultz, 1947).

How this variant initially arose cannot be stated, of course. It is possible to speculate on this, however. We know that in this instance, at least, the organism had persisted in the lesions of the patient for some months and that the organism had access to fat, and possibly to glycerol as such. In such an environment the organism certainly enjoyed an opportunity to make use of these substances. It is known, of course, that in the presence of a given substrate organisms may

acquire the ability to attack or utilize that substrate (adaptive enzymes). Such facility might conceivably be acquired by relatively few individuals in the total population and, once acquired, might be retained for a considerable period of time, especially in the presence of a continued opportunity to exercise the special function. We have seen above that, although the gelatinous forms are in the main stable, there is nevertheless a tendency for reversions to nongelatinous forms to occur. This is more marked on media that do not contain glycerol. Even under these conditions, however, a remarkable tenacity is exhibited in the retention of this physiological property, bordering on what one would expect of a true mutant.

The fact that another such variant was isolated from an ordinary laboratory stock strain suggests that the property can be acquired by other strains as well. This particular stock strain was isolated from an infection in 1934, but during the past five years or so it has been carried on glycerol agar, which was found to favor pigment production by different strains. Whether the gelatinous variant has persisted through all these years since the strain was isolated or whether it arose in response to the later culturing on glycerol agar, it is, of course, impossible to say, but it seems possible that the opportunity to utilize glycerol was a 'actor in the appearance of this variant.

The exceedingly large size attained by gelatinous colonies can be fully accounted for by the gelatinous material that is laid up within them. A remarkable feature of the growth of these colonies is that they continue to increase in size over a very much longer period of time than do ordinary colonies. How much of this growth is due to continued multiplication of the organisms or to mere synthesis of more and more of the gelatinous material is hard to determine. Most of the ultimate size of these colonies is undoubtedly due to the gelatinous material that is laid up. It would seem to be continuously synthesized by organisms still in close contact with the substrate; but, as has been pointed out, collections of organisms may be demonstrated throughout the colony, whatever may be the role that these play in the synthesis of this gelatinous material as growth of the colony continues. It seems probable that the organisms that are more removed from the medium have been carried upward by the gelatinous material produced and have ceased to take part in the further production of this material.

It should be emphasized that these gelatinous variants are definitely jellylike and are not to be confused with "mucoid" colony variations so frequently encountered in bacteriological work. There is nothing stringy or viscid about this material. In its most characteristic form it is like a stiff jelly, and the original colonies (pearl forms) resembled firm beads planted on the surface of the medium. In certain of the subvariants that have arisen from the original clones the material produced has become more fluid, but still not sticky or mucoid.

It is not difficult to account for the differences in shape or surface contours of the gelatinous colonies. These appear to be rooted in the variations in colony form commonly observed in nongelatinous colonies of this species. These range from convex or umbonate to highly rugose forms. What we have in the case of

the gelatinous forms is merely a gelatinous matrix superimposed on these ordinary colony variants. In other words, the ability to utilize glycerol for the production of this gelatinous material is not associated with any particular basic colony form. Whatever changes occur in the latter become associated with corresponding shifts in the configuration of the gelatinous forms. There is on the whole a rather marked tendency for the usual convex forms to become rugose forms, so that the gelatinous forms that originally occurred as typical pearl forms on glycerol media have become much less frequent than they were in the beginning. Fiala (1941) has pointed out that glycerol hastens the induction of rugose forms.

It should probably be mentioned also that the gelatinous variation is not only identified with the different basic variations in colony form exhibited by *P. aeruginosa*, but with other variations that may be exhibited by this organism, such as occur in pigment production. It is possible therefore to have gelatinous forms in which pyocyanin production is pronounced and others in which there is little or no pyocyanin production; this applies also to the fluorescent pigment. The color of these gelatinous colonies has therefore varied widely—from almost white or light amber through shades of red to a deep green. Observations have also been made which suggest that this variation is independent of certain other physiological variations known to occur within this species. We were primarily concerned, however, with the variation that resulted in the production of the gelatinous material.

There is undoubtedly some physical difference in the gelatinous material produced by the variant on different media. This is evident from differences in its consistency when it is grown on different substrates. Moreover, the fact that certain compounds other than glycerol were utilized to produce the jellylike substance may mean that the variant possesses enzymes necessary for synthesizing more than one such substance. This could not be investigated in the time available.

Whether the alveolar structure seen in the sections of colonies pre-exists or is an artifact produced by contact of the sections with alcohol in dehydration is difficult to determine. It could well be due to the latter. The finer strands correspond to the fine fibers formed when an aqueous solution of gelatinous material is precipitated with alcohol. It is possible that the intact gelatinous colony is a fairly homogeneous mass rather than alveolar in structure.

The serological work was conducted primarily to determine whether the gelatinous material possessed antigenic properties that might perchance confer a degree of specificity to the variant. No evidence of this was obtained.

SUMMARY

A new variant of *Pseudomonas aeruginosa* has been identified. It is characterized by the production of exceedingly large jellylike colonies when grown on media containing glycerol. The variation seems to consist wholly of ability to produce large amounts of gelatinous material and appears to be entirely independent of other variations exhibited by this organism. The nature of the ge-

latinous material has not been fully determined, but it appears to be a carbohydrate of some kind. Similar gelatinous colonies are produced by the variant when it is grown in the presence of certain alcohols other than glycerol, and in the presence of certain oils or fats. However, the nature of the material produced on these latter substrates has not been compared chemically with that produced on glycerol.

The variation appears to be wholly physiological in nature and may be superimposed on any of the basic colony types of the organism. The variant is, in the main, stable, but some reversion to ordinary nongelatinous colonial forms does occur, especially when it is grown for a time in fluid media free of glycerol. The nongelatinous colonies which then appear as a rule correspond topographically to the gelatinous forms except for the absence of the gelatinous material. The gelatinous forms show about the same range of variation in pigment production observed in ordinarily nongelatinous colonial forms. They are, moreover, not distinguishable from ordinary nongelatinous forms on the basis of serological or phage susceptibility tests.

Sections prepared from large gelatinous colonies show that the material in these colonies consists primarily of gelatinous material, with relatively few organisms irregularly distributed through the material. It seems probable, however, that the material is synthesized primarily by organisms located next to the medium and that most of the organisms found in the upper strata have been carried upward as the material is produced.

The consistency of the gelatinous material produced varies to some extent among different gelatinous clones. Although like a stiff jelly as a rule, it may be relatively thin or watery in the case of certain clones. It is, however, in no sense viscid and is therefore not to be confused with a mucoid type of variation.

Although this particular variation appears to be of infrequent occurrence, it should be kept in mind that media containing glycerol have probably been rarely employed in the initial isolation of the organism from infected lesions. However, in addition to the variant originally isolated from an infected lesion, we have isolated a similar variant from a stock strain being carried on a glycerinated medium.

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THE NUTRITIONAL REQUIREMENTS OF HEMOPHILUS PARAINFLUENZAE 7901[1]

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A complex mixture of known nutrients was utilized as a basal medium in the identification of putrescine as an essential growth factor for *Hemophilus parainfluenzae* ATCC 7901 (Herbst and Snell, 1948, 1949). By the addition of an optimum concentration of putrescine to this medium it has been possible to determine the remaining nutritional requirements of this organism, and to develop a simplified synthetic medium suitable for its growth. Details of these studies are presented here.

METHODS

The basal medium is shown in table 1. The growth obtained following omission of one or more components from this medium was compared with the growth response in the intact basal medium. Growth equal or superior to that obtained with the complete medium was considered necessary to demonstrate that a given component of the medium was nonessential for growth. Cultural methods were identical to those previously described (Herbst and Snell, 1949). Growth was measured turbidimetrically in the Evelyn colorimeter after 38 hours of incubation at 37 C.

RESULTS

Amino acid requirements. The growth response of *H. parainfluenzae* following the omission of individual amino acids from the complete medium is shown in table 2. Growth was severely depressed by the omission of either arginine, isoleucine, valine, or tyrosine from the amino acid mixture; the single omission of cystine and glutamic acid caused a less pronounced depression of growth. Omission of any of the amino acids not indicated in the table failed to affect growth adversely. When a simplified mixture that contained only these essential amino acids was prepared, it failed to support growth equivalent to that given by the more complete medium (table 3). Under these conditions, additional amino acids, as shown in the table, were stimulatory (or essential) for growth. Apparently the synthesis of the latter amino acids becomes limiting when only the simplified mixture is supplied, although it is not when these amino acids are omitted individually from a complete mixture of amino acids.

The vitamin requirements. The vitamin requirements of *H. parainfluenzae* were

TABLE 1

*Composition of the basal medium**

COMPONENT	AMOUNT PER 10 ML	COMPONENT	AMOUNT PER 10 ML	COMPONENT	AMOUNT PER 10 ML
DL-Aspartic acid	10 mg	L-Cystine	1 mg	Inositol	200 μg
L-Glutamic acid	10 mg	L-Tyrosine	1 mg	Biotin	0.01 μg
DL-Alanine	10 mg	Glycine	1 mg	p-Aminobenzoic acid	0.01 μg
L-Arginine·HCl	2 mg	Glucose	10 mg	Folic acid	0.1 μg
DL-Methionine	2 mg	Sodium acetate	60 mg	Coenzyme I	1 μg
L-Leucine	1 mg	Guanine hydrochloride	100 μg	$MgSO_4 \cdot 7H_2O$	1 mg
DL-Threonine	2 mg	Adenine sulfate	100 μg	$CaCl_2 \cdot 2H_2O$	400 μg
DL-Serine	2 mg	Uracil	100 μg	$FeSO_4 \cdot 7H_2O$	135 μg
		Putrescine dihydrochloride	16 μg		
L-Proline	1 mg	Thiamine chloride	1 μg	$ZnSO_4 \cdot 7H_2O$	4 μg
DL-Tryptophan	2 mg	Riboflavin	1 μg	$CuSO_4 \cdot 5H_2O$	4 μg
DL-Valine	2 mg	Nicotinic acid	5 μg	$CoCl_2 \cdot 6H_2O$	4 μg
DL-Phenylalanine	2 mg	Nicotinamide	5 μg	$MnSO_4 \cdot H_2O$	3 μg
L-Histidine	1 mg	Pyridoxine·HCl	20 μg	K_2HPO_4	15.6 mg
DL-Isoleucine	2 mg	Calcium pantothenate	10 μg	KH_2PO_4	1.4 mg
L-Lysine	2 mg	Choline chloride	50 μg		

* Ten ml of this medium were inoculated with 0.1 ml of a washed, 12-hr inoculum, diluted to a turbidity of 95 (Evelyn colorimeter). The culture receptacles were 150-ml pyrex milk dilution bottles. The medium was adjusted to pH 7.8 with NaOH and sterilized by autoclaving for 15 minutes at 120 C. Coenzyme I was sterilized by Seitz filtration and added to the cooled sterile medium (see Herbst and Snell, 1949, for further details of procedure).

TABLE 2

Amino acid requirements of H. parainfluenzae

OMISSION FROM COMPLETE MEDIUM	TURBIDITY*
None	80
L-Arginine hydrochloride	94
DL-Isoleucine	99
DL-Valine	99
L-Tyrosine	95
L-Glutamic acid	87
L-Cystine	89
L-Leucine	80
L-Lysine	79
DL-Phenylalanine	80
DL-Aspartic acid	80

* Per cent of incident light transmitted; uninoculated medium = 100.

found to be relatively simple and not at all unusual (Peterson and Peterson, 1945). Omission of either biotin or calcium pantothenate from the vitamin mixture resulted in complete growth failure. The effects of omitting thiamine and

pyridoxine were less pronounced, but normal growth was not obtained in their absence, and vitamin B_6 appears essential when a restricted mixture of vitamins

TABLE 3

Stimulatory effect of "nonessential" amino acids when added to a minimal mixture

AMINO ACIDS IN MEDIUM	TURBIDITY*
(1) "Complete" mixture of basal medium (table 1)..	82
(2) Simplified mixture of essential amino acids (table 2)†.	95
(3) Same as (2) + L-leucine	88
(4) Same as (3) + DL-phenylalanine	85
(5) Same as (4) + L-lysine	82
(6) Same as (5) + DL-aspartic acid	80

* Per cent of incident light transmitted; uninoculated medium = 100.

† L-Arginine, DL-isoleucine, DL-valine, L-tyrosine, L-glutamic acid, and L-cystine at the concentrations indicated in table 1.

TABLE 4

The vitamin requirements of H. parainfluenzae

VITAMIN MIXTURE IN MEDIUM	TURBIDITY*
(1) "Complete" mixture of basal medium (table 1)	76
(2) Same as (1) minus thiamine chloride.	79
(3) Same as (1) minus calcium pantothenate	100
(4) Same as (1) minus biotin.	100
(5) Same as (1) minus pyridoxine hydrochloride...	77
(6) Thiamine chloride, calcium pantothenate, and biotin. . .	97
(7) Same as (6) + pyridoxine hydrochloride	76

* Per cent of incident light transmitted; uninoculated medium = 100.

TABLE 5

The effect of uracil on the growth of H. parainfluenzae

ADDITIONS PER 10 ML OF MODIFIED MEDIUM*	TURBIDITY†
None	97
100 μg adenine sulfate, guanine hydrochloride, and uracil......	76
1 μg uracil..	96
5 μg "	89
10 μg "	81
25 μg "	78
50 μg "	76
100 μg "	77

* Basal medium (table 1) minus purine bases and uracil.

† Per cent of incident light transmitted; uninoculated medium = 100.

is used (table 4). The remaining vitamins of the basal medium were neither essential nor stimulatory for growth.

Purine and pyrimidine bases. Almost complete growth failure resulted when

adenine, guanine, and uracil were omitted from the basal medium (table 5). However, maximum growth could be obtained in the absence of the purine bases if an optimum concentration of uracil (25 to 100 μg per 10 ml of medium) was added to the basal mixture. The specificity of this requirement is shown in table 6. Cytosine and thymine could not be utilized as substitutes for uracil, either

TABLE 6

A comparison of compounds effective in supplying the pyrimidine requirement of H. parainfluenzae

COMPOUND*	REQUIREMENT FOR HALF-MAXIMUM GROWTH	
	μg/10 ml	μM/10 ml
Uracil...	7	0.069
Uridine..	14	0.060
Uridylic acid......................................	19	0.055
Cytidine...	10	0.041
Cytidylic acid.............................	16	0.050
Thymidine...	>250	>1.0
Desoxycytidine....................................	20	0.090

* For the basal medium used, see first footnote of table 5.

TABLE 7

The effect of purine bases on the growth of H. parainfluenzae in media containing uracil*

ADDITIONS TO MODIFIED BASAL MEDIUM†	TURBIDITY‡
None..	70
Adenine sulfate and guanine hydrochloride....................	70
Adenine sulfate..	85
Guanine hydrochloride......................................	94
Hypoxanthine...	73
Xanthine..	74
Adenine sulfate and hypoxanthine............................	73
Adenine sulfate and xanthine................................	84
Guanine hydrochloride and hypoxanthine......................	74
Guanine hydrochloride and xanthine..........................	96

* Basal medium (table 1) minus adenine and guanine (contains 100 μg of uracil per 10 ml).

† One hundred μg of each compound added to 10 ml of medium.

‡ Per cent of incident light transmitted; uninoculated medium = 100.

in the presence or absence of the purine bases. However, cytidine, cytidylic acid, uridine, and uridylic acid were utilized as efficiently as was uracil. Thymidine showed very low growth-promoting activity; the requirement for half-maximum growth was 20 to 30 times that of the active compounds. This slight activity may possibly result from impurities in the preparation used. By contrast, the desoxyriboside of cytosine was highly active. Uracil was most active on a weight basis; however, on a molar basis cytidine, cytidylic acid, uridylic acid, and uridine were all slightly more active than uracil.

The single addition of either guanine or adenine to media containing uracil inhibited growth (table 7). When both were added together, however, no inhibition was apparent. Hypoxanthine and xanthine were not inhibitory under the same conditions, and hypoxanthine, but not xanthine, effectively overcame the inhibitory effects of adenine and guanine. These interesting relationships are highly reproducible and are somewhat similar to those observed in other organisms (e.g., Pennington, 1942; Loring and Pierce, 1944); their explanation, however, is not apparent at the present time.

Composition of a simplified medium. The information obtained in the foregoing experiments permitted the development of the simplified medium of table 8. The inorganic nutrition of the organism has not been critically examined in media rigorously freed of individual ions. However, minor changes in the salts

TABLE 8

*Composition of a simplified synthetic medium for H. parainfluenzae 7901**

COMPONENT	AMOUNT PER 10 ML	COMPONENT	AMOUNT PER 10 ML
L-Glutamic acid	10 mg	Uracil	100 μg
DL-Aspartic acid	10 mg	Putrescine dihydrochloride	16 μg
L-Arginine hydrochloride	2 mg	Thiamine chloride	10 μg
DL-Isoleucine	2 mg	Calcium pantothenate	10 μg
DL-Valine	2 mg	Biotin	0.01 μg
L-Cystine	1 mg	Pyridoxine hydrochloride	20 μg
L-Tyrosine	1 mg	Coenzyme I	1 μg
L-Leucine	1 mg	$CaCl_2 \cdot 2H_2O$	40 μg
L-Lysine	2 mg	$MgSO_4 \cdot 7H_2O$	1 mg
DL-Phenylalanine	2 mg	$FeSO_4 \cdot 7H_2O$	135 μg
Glucose	10 mg	$NaNO_3$	10 mg
Sodium acetate	60 mg	K_2HPO_4	31.2 mg
		KH_2PO_4	2.8 mg

* The cultural conditions are described briefly in table 1, and in detail by Herbst and Snell (1949).

mixture of the original basal medium (table 1) have been made as a result of experiments that are not given in detail. The Zn^{++}, Cu^{++}, Co^{++}, and Mn^{++} of the basal medium were neither essential nor stimulatory and were omitted from the simplified mixture. The Mg^{++}, Fe^{++}, and Ca^{++} salts were retained since the omission of any one of them resulted in slightly reduced growth. The Ca^{++} level was reduced to avoid formation of the undesirable inorganic precipitate in the original medium during autoclaving. The concentration of K_2HPO_4 and KH_2PO_4 was doubled since the higher level gave uniformly better results with the simplified medium. $NaNO_3$ was added since it stimulated growth very slightly in several experiments. Glucose and sodium acetate were essential for maximum growth in either medium.

The growth of H. parainfluenzae in a simplified medium. The response of *H. parainfluenzae* to putrescine in the simplified medium and in the original basal

medium (both minus putrescine) is compared in figure 1. Low concentrations of putrescine are considerably less effective in promoting growth in the simplified medium than in the complex medium. However, the maximum growth obtained with excess putrescine is similar in the two media.

The cause of this decreased response to low concentrations of putrescine is not fully known. Presumably, the increased demands placed on the bacteria grown in the less complex medium for the synthesis of a number of essential metabolites have increased the requirement for putrescine. However, the addition (singly) of those compounds absent from the simplified medium did not improve growth of the organism. Similarly, the single addition of either the complete vitamin mixture, the amino acid mixture, or the purine bases did not enhance the maximum growth obtained with an excess of putrescine.
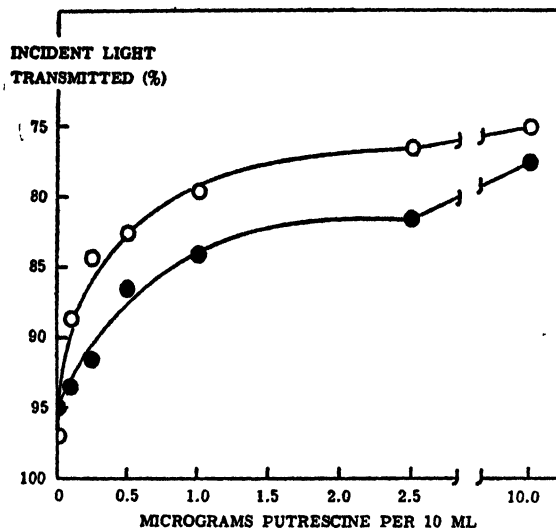
Figure 1. The comparative response of H. parainfluenzae to putrescine in the original (O) and simplified (●) medium.

DISCUSSION

The nutritional requirement of H. parainfluenzae for (a) coenzyme I (Lwoff and Lwoff, 1937) and (b) putrescine (or its congeners, spermine or spermidine —Herbst and Snell, 1948, 1949) are quite unusual. Several Hemophilus species require coenzyme I (Knight, 1945), but no other organisms having this requirement are known. In all cases examined, however, bacteria which do not require the pyridine nucleotides as essential nutrients can synthesize them. Perhaps putrescine represents an additional requirement common to many organisms of the Hemophilus group. This possibility does exist since only H. pertussis (Hornibrook, 1940) and H. parainfluenzae have been cultured in purified media.[2]

[2] A requirement of putrescine for H. pertussis is not eliminated, since growth of this organism in Hornibrook's medium was stimulated by the addition of hydrolyzed casein or of yeast extract. Both supplements contain putrescine (or spermine or spermidine), as indicated by assay with H. parainfluenzae 7901.

The specificity of the requirement of *H. parainfluenzae* for uracil is very similar to that of *Tetrahymena geleii* (Kidder and Dewey, 1948) and to that of uracilless *Neurospora* mutants (Loring and Pierce, 1944). For *Tetrahymena geleii*, as for *H. parainfluenzae*, cytidine, cytidylic acid, uridine, and uridylic acid were, on the molar basis, roughly equivalent to uracil in activity, but cytosine was inactive. For certain strains of uracilless *Neurospora*, however, uridine and cytidine were many times more active than uracil in promoting growth. Here again, cytosine was inactive. These similarities in widely diverse organisms point to a common mechanism for the synthesis and interconversion of pyrimidines and pyrimidine nucleosides.

An antagonism between purine bases has previously been reported in nutritional investigations with bacteria (Pennington, 1942) and molds (Fairley and Loring, 1949). Pennington observed that if the ratios of adenine to hypoxanthine or of guanine to hypoxanthine were high, the growth-promoting effect of hypoxanthine for *Spirillum serpens* was completely masked. Neither adenine nor guanine alone was active in promoting growth, but a mixture of approximately equal parts of the two would replace hypoxanthine. Fairley and Loring showed a definite inhibition of the growth of a purine-deficient strain of *Neurospora* when the ratio of guanine to hypoxanthine was greater than one. These results, though similar, are not entirely comparable to the purine antagonisms observed in *H. parainfluenzae* since the latter organism, in contrast to *S. serpens* and the mutant *Neurospora* discussed above, grows in the absence of added purine bases, i.e., is able to synthesize them. However, the cases cited do emphasize the extent to which imbalances in the medium employed may affect the apparent nutritional requirements of a given test organism.

SUMMARY

A simplified synthetic medium has been developed which supports growth of *Hemophilus parainfluenzae* 7901.

Arginine, isoleucine, valine, tyrosine, glutamic acid, cystine, leucine, phenylalanine, lysine, and aspartic acid are essential for maximum growth of this organism. Biotin, calcium pantothenate, thiamine, and vitamin B_6 are essential vitamins. Coenzyme I (or II) and putrescine (or spermine or spermidine) are also essential. A suitable pyrimidine is essential for growth; uracil, uridine, uridylic acid, cytidine, cytidylic acid, or desoxycytidine can serve in this role. Cytosine is inactive. Glucose and sodium acetate are also required for maximum growth.

Growth of *H. parainfluenzae* in the synthetic medium is severely inhibited by the single addition of either adenine or guanine. Such inhibition of growth is alleviated by the simultaneous addition of hypoxanthine, or of appropriate combinations of other purine bases.

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STUDY ON TOXINS AND ANTIGENS OF SHIGELLA DYSENTERIAE

II. Active Protection of Rabbits with Whole Organisms and Fractions of Shigella dysenteriae

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Bacteria contain a variety of components that may give rise to antibodies devoid of protective power. In prophylactic immunization the presence of such useless antigens ideally should be avoided. Not only is the production of non-protective antibodies an unnecessary load, but, in some cases, particularly that of streptococci, it may be unfavorable and even dangerous. Although there is evidence of protection induced by the administration of whole-organism vaccines, the untoward toxic effects of killed bacteria and the sensitivities that may be induced by some of the components of the organisms entirely unrelated to protection make the search for purified protective antigens necessary (Dubos, 1946).

In the case of *Shigella dysenteriae*, the problem is further complicated by the claims of various investigators that different fractions of the organisms induce different protective antibodies. In the case of *S. dysenteriae*, the proponents of the two-toxin theory maintain that antibodies produced in response to the injection of the neurotoxin protect the animals against the toxin alone, and that antibacterial immunity is afforded solely by immunization with the whole smooth variant of *S. dysenteriae* or with its somatic antigen (Boivin and Mesrobeanu, 1937; Morgan and Partridge, 1941; Steabben, 1943). In a previous publication (Boroff, 1949) we have contended that there exists only one dominant antigen in *S. dysenteriae* and that the single toxin elaborated by this organism is associated with it. If this contention is true, it should also hold true that protective antibodies against one fraction of *S. dysenteriae* should protect the immunized animals against all fractions as well as against whole organisms. The following report demonstrates the active protection of rabbits by means of whole organisms and variously derived fractions of *S. dysenteriae*.

EXPERIMENTAL PROCEDURES AND RESULTS

Immunization of rabbits with S and R variants of S. dysenteriae. Smooth and rough variants of *S. dysenteriae*, strain 2308, obtained from Dr. R. Dubos, were used throughout this study. The smooth variant agglutinated with sera against all strains of *S. dysenteriae* on hand. The rough variant agglutinated neither with undiluted antisera to the smooth variant, nor with sera of rabbits immunized with the rough strains.

The organisms were grown in Roux bottles on a 2 per cent tryptone agar con-

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taining 0.1 per cent glucose and 0.1 per cent sodium chloride. The cultures were incubated at 37 C for 18 hours, and the growth was washed off with saline solution. After centrifugation, the sediment was washed twice, resuspended in saline solution, and dried by the lyophil process. All antigens described in this report were prepared from lyophilized organisms.

Since the rough variant of *S. dysenteriae* lacked somatic antigen and was nevertheless toxic according to the above-mentioned authors, the protection afforded by immunization with this variant should be directed only against the "neurotoxin," whereas injections with the smooth variant should afford antibacterial immunity. However, on the basis of our hypothesis that there is only one dominant toxin in *S. dysenteriae*, it should follow that the injection of any antigenic fraction of this organism, capable of inducing protective antibodies, will afford protection against the toxic effects of the whole organism as well as the purified toxin. These alternatives were tested by the immunization of two groups of six rabbits each with smooth and rough heat-killed variants of *S. dysenteriae*.

The organisms were suspended in sterile saline solution and heated in a water bath for 30 minutes at 56 C. If no viable organisms could be demonstrated upon subsequent tests, the suspensions were used for injections. The rabbits were given 6 intravenous injections on alternate days. The total amount injected was 57 micrograms (dry weight) of the organisms. Five days after the last injection the rabbits were bled and their sera tested for the presence of agglutinins and precipitins. Two days after the bleeding the rabbits were challenged with 1 mg of the homologous antigen. Most rabbits survived the initial challenge, but all of the normal controls died within 48 hours.

After being observed for 5 days the surviving rabbits were injected with partially purified toxin obtained from the smooth variant by Dubos' method (1946) described below. Table 1 summarizes the results. All animals injected with the toxin survived, showing that they were protected not only against whole organisms but purified toxin as well. A point of great interest was the observation that although the rabbits immunized with the rough variant showed no demonstrable antibodies, they were protected against lethal doses of smooth whole organisms and purified toxin.

Immunization of rabbits with sonic lysates of smooth and rough variants of S. dysenteriae. One gram each of lyophilized organisms of the smooth and rough variants of *S. dysenteriae* was resuspended in 40 ml of distilled water and treated in the magnetostriction oscillator for 1½ hours at 9,000 cycles per second. The treated material was centrifuged to remove the remaining organisms and debris, and the supernatant fluid filtered through a Berkefeld candle. The filtrate was a clear greenish fluid which upon lyophilization formed a white feathery substance that was readily soluble in saline solution. This substance was as toxic for mice on intraperitoneal injection as were intact organisms.

Six rabbits were injected with a saline solution of each of the sonic lysates. Each rabbit received 0.057 mg of the substance intravenously in 6 injections, over a period of 2 weeks. Unless otherwise stated, the injection schedules for all

antigens were as follows: The first dose was 0.002 mg of antigen per rabbit, the second dose 0.005 mg, the third and the fourth 0.01 mg each, and the fifth and sixth 0.02 mg each.   Five days after the last injection the sera of the rabbits were tested for agglutinins and precipitins.   All rabbits immunized with the smooth lysate showed the presence of these antibodies, whereas rabbits immunized with rough lysate did not.   Survival of these rabbits after the injection with 20 LD_{50} of the homologous antigen proved that they were protected against this dose of toxin.   A second challenge with a lethal dose of intact organisms showed that all rabbits were as resistant to whole organisms as they were to the lysates.   The results of this experiment are also shown in table 1.

TABLE 1

Serological reactions and degree of active protection exhibited by rabbits immunized with whole organisms and various fractions of S. dysenteriae

RABBITS IMMUNIZED WITH	HIGHEST SERUM DILUTION GIVING COMPLETE AGGLUTINATION WITH S2308 WO	DEGREE OF PRECIPITATION REACTION WITH ANTIGEN DILUTIONS OF		SURVIVAL RATIO OF RABBITS CHALLENGED WITH 20 LD_{50} OF HOMOLOGOUS ANTIGEN	SURVIVAL RATIO OF RABBITS CHALLENGED WITH HETEROLOGOUS ANTIGEN
		1:1,000	1:10,000		
S2308 WO	1:1,024	4	3	4/6	4/4
R2308 WO	0	0	0	6/6	6/6
S2308-b	1:1,024	4	3	6/6	6/6
R2308-b	0	0	0	6/6	6/6
S2308 PT	1:1,024	4	3	5/6	5/5
R2308 PT	0	0	0	4/6	4/4
S2308 SA	1:1,024	4	0	5/6	0/5
R2308 SA	0	0	0	6/6	0/6

S, smooth variant.   WO, whole heat-killed organisms.
R, rough variant.   PT, partially purified toxin.
b, sonic lysate.   SA, somatic antigen.
    Partially purified toxin, hydrochloric acid precipitate from sonic lysate of smooth and rough cultures of *S. dysenteriae*.
    Heterologous antigen challenge:
S2308 WO and R2308 WO—challenged with S2308 partially purified toxin.
S2308-b, R2308-b, S2308 PT, and R2308 PT—challenged with S2308 WO.
S2308 SA—challenged with S2308 WO.
R2308 SA—challenged with S2308 partially purified toxin.

Immunization of rabbits with partially purified toxin from smooth and rough variants of S. dysenteriae.   The preceding experiment established that rabbits immunized with smooth and rough variants of *S. dysenteriae* will withstand lethal doses of the partially purified toxin.   This experiment attempted to determine the immunizing potency of the purified toxin from these variants.
    The toxin was obtained by the disintegration of 1 gram of dry weight of organisms suspended in distilled water in the magnetostriction oscillator at 9,000 cycles per second.   The solution of the bacterial substance was centrifuged at 10,000 rpm to remove the remaining intact organisms and debris, and the clear supernatant fluid was filtered through a Berkefeld candle.   The toxin was separated from the solution, according to the method of Dubos and Geiger (1946), by

precipitation with N/1 hydrochloric acid at pH 4.0 at 4 C. The precipitate was washed in acidified water, redissolved in distilled water with the aid of N/1 NaOH, and dialyzed against cold distilled water overnight. The solution was dried *in vacuo* and the dried material tested for toxicity in mice.

Fifty-seven micrograms each of toxin obtained from the smooth or rough variants of *S. dysenteriae* were injected intravenously into rabbits. Six rabbits were immunized with each preparation. Each rabbit received 6 injections given on alternate days. Five days after the last injection the rabbits were tested for the presence of circulating antibodies. Rabbits injected with toxin from the rough variant showed neither agglutinins nor precipitins. The rabbits injected with toxin from the smooth variant possessed these antibodies. The animals in both groups were then challenged with 1 mg of the homologous toxin and all survived. Five days after the first challenge each rabbit was injected with 1 mg of whole dried organisms. All rabbits survived the second challenge as well, showing that the partially purified toxin from either rough or smooth variant afforded protection against the smooth whole organism possessing presumably the toxic somatic antigen. The results are summarized in table 1.

Immunization of rabbits with diethylene glycol extracts of smooth and rough variants of S. dysenteriae. Morgan (1937) extracted a smooth strain of *S. dysenteriae* with anhydrous diethylene glycol and obtained a substance that upon chemical analysis proved to be a polypeptide-carbohydrate-lipoid complex. Rabbits injected with this substance were shown to possess in their sera agglutinins and precipitins against the homologous strain or its products. Morgan termed this substance the somatic antigen of *S. dysenteriae*. This substance was toxic for mice in 0.25-mg amounts.

Using Morgan's method, diethylene glycol extracts were obtained both from the smooth variant and the rough variant of strain 2308. After 5 days of extraction in the cold with frequent shaking, the diethylene glycol was removed by dialysis and the resultant colloidal suspension was precipitated with 66 per cent cold acetone. The precipitate was washed with acetone and alcohol and dried *in vacuo*. This substance readily resuspended in water and in this state was used for the immunization of rabbits. The method of injection and the dosages were similar to those used in the immunization with whole organisms and purified toxins.

Tests for circulating antibodies revealed that animals injected with extracts from smooth organisms possessed in their sera agglutinins against whole smooth organisms and precipitins against purified toxin from these organisms as well as the homologous antigen. Rabbits injected with the diethylene glycol extract of rough organisms possessed no such antibodies. However, neither the former nor the latter group of animals was protected against the challenge with 1 mg of smooth *S. dysenteriae*. The results of this experiment are also summarized in table 1.

Immunization of rabbits with a nontoxic variant of S. dysenteriae 2308. A nontoxic variant of the smooth strain 2308 of *S. dysenteriae* was obtained by growing a 7-hour seed culture of the toxic strain in 15 liters of veal infusion broth at pH

7.0 in a steel tank. The culture was grown for 18 hours with vigorous shaking. A growth of 10 billion organisms per ml was obtained. The organisms were separated from the medium by centrifugation, washed three times with saline solution, and lyophilized. Mice survived the intraperitoneal injection of 1 mg (dry weight) of this variant. A suspension of these organisms agglutinated, however, with all the anti-whole-organism sera on hand. Therefore, it was decided to test the protection afforded by immunization of rabbits with this nontoxic strain.

Three rabbits were given 3 intravenous injections, each consisting of 1 mg of heat-killed organisms. Five days after the last injection, the sera of these rabbits were tested for circulating antibodies. All sera showed agglutinins and precipitins. Table 2 shows the results obtained. Three days after the test bleeding, each rabbit received 2 mg of toxic organisms of the homologous strain. All rabbits survived.

Active protection afforded by immunization of rabbits with detoxified whole organisms and sonic lysates of S. dysenteriae. An earlier article (Boroff, 1949) described the detoxification of various preparations from *S. dysenteriae* by treat-

TABLE 2

Serological reactions and active protection exhibited by rabbits immunized with nontoxic strain of S2308 S. dysenteriae

RABBIT NO.	HIGHEST SERUM DILUTION GIVING COMPLETE AGGLUTINATION*	DEGREE OF PRECIPITATION REACTION WITH ANTIGEN DILUTIONS† OF		RESULT OF CHALLENGE WITH 40 LD₅₀ OF TOXIC STRAIN 2308
		1:1,000	1:10,000	
1	1:1,024	4	3	Survived
2	1:1,024	4	3	Survived
3	1:1,024	4	1	Survived

* Test antigen in agglutination test, S2308 heat-killed organisms.
† Test antigen in precipitation test, S2308 partially purified toxin.

ment with ketene gas. It was shown that none of the antigenic power of these preparations was lost by this treatment and that rabbits could withstand the injection of as much as 1 mg of detoxified preparations.

To test whether rabbits could be protected against untreated preparations by injection of the detoxified antigens, six rabbits were injected intravenously with detoxified whole organisms and six with detoxified sonic lysate. Each rabbit received 57 micrograms of the respective preparation over a period of 2 weeks. Five days after the last injection the rabbits were bled and their sera titrated for agglutinins and precipitins. The results are recorded in table 3.

All rabbits showed the presence of agglutinins and precipitins. However, when challenged 2 days after the bleeding with untreated homologous preparations of whole organisms and sonic lysates, none of the rabbits survived. Apparently ketene treatment not only destroyed the toxicity of the whole organism and sonic lysate of *S. dysenteriae*, but also affected the antigen responsible for the formation of protective antibodies.

Duration of immunity in rabbits immunized with whole organisms and various

fractions of S. dysenteriae. It has been observed during the immunization of rabbits for the purpose of antiserum production that the animals could withstand, at the end of the immunization schedule, injections of the toxic material in excess of 20 LD_{50} of the antigen. In order to determine whether this active protection would last, the rabbits were tested with 20 LD_{50} of the homologous antigen 6 months after the last immunizing injection. The number of rabbits and the antigens used as well as the outcome of the challenge are shown in table 4.

TABLE 3

Serological reactions and active protection exhibited by rabbits immunized with whole organisms and sonic lysate detoxified by ketene

RABBITS IMMUNIZED WITH ACETYLATED	HIGHEST SERUM DILUTION GIVING COMPLETE AGGLUTI- NATION*	DEGREE OF PRECIP- ITATION REACTION WITH ANTIGEN DILUTIONS† OF		SURVIVAL RATIO OF RABBITS CHALLENGED WITH 20 LD_{50} OF HOMOLOGOUS UNTREATED ANTIGEN
		1:1,000	1:10,000	
Smooth whole organisms 2308	1:1,024	4	3	0/6
Sonic lysate of smooth whole organisms 2308.	1:1,024	4	3	0/6

* Test antigen in agglutination test—S2308 WO.
† Test antigen in precipitation test—S2308 partially purified toxin.

TABLE 4

Active protection shown by rabbits immunized with whole organisms and various fractions of smooth and rough variants of S. dysenteriae six months after immunization

NUMBER OF RABBITS IMMUNIZED	RABBITS IMMUNIZED WITH	RABBITS CHALLENGED WITH	SURVIVAL RATIO OF RABBITS CHALLENGED WITH 20 LD_{50} OF ANTIGEN
10	S2308 WO	S2308 WO	0/10
8	S2308-b	S2308-b	3/8
3	S2308 PT	S2308 PT	0/3
3	R2308 PT	R2308 PT	0/3
3	S2308 SA	S2308 WO	0/3
5	R2308 SA	R2308 WO	0/5

S, smooth variant.
R, rough variant.
Purified toxin, trichloracetic-acid-precipitated autolyzate of S and R variants of *S. dysenteriae.*
Somatic antigen, diethylene glycol extract from S and R variants of *S. dysenteriae.*

It is apparent that only a few rabbits retained their immunity at the end of 6 months.

DISCUSSION

The data obtained in active protection experiments indicate that it is possible to immunize rabbits with either smooth or rough whole organisms or with their respective toxins and thus induce active protection against all of these substances.

Not only whole organisms or solutions of whole organisms afforded protection against each other, but chemically purified toxins protected the animals and induced the formation of identical and reciprocally absorbable antibodies.

This phenomenon of cross protection is not in accord with the concept that two toxins and two distinct antigens exist in *S. dysenteriae*. It is, however, explainable on the basis of the presence of one dominant antigen in the organism. Futhermore, the protection afforded rabbits against the whole organism of the smooth variant by immunization with a sonic lysate of the rough variant lends additional support to the latter hypothesis. The absence of agglutinins and precipitins obviously does not denote lack of protection against *S. dysenteriae*, for it has been observed that rabbits immunized with the rough variant of *S. dysenteriae* or toxic substances derived from this variant, although showing no agglutinins or precipitins in their sera, were, nevertheless, protected against lethal doses of either smooth whole organisms or the purified toxins of this variant.

The protection afforded by immunization of rabbits with rough organisms or their fractions need not, however, be ascribed to the presence of antitoxin. No toxin-antitoxin flocculation could be observed, although both antirough serum and antigen concentrations were tested over a wide range. Neither did the addition of rough toxin to smooth antitoxin inhibit subsequent flocculation with smooth variant toxin. The phenomenon of eliciting protection in the absence of demonstrable circulating antibody has been observed with organisms devoid of exotoxin. Dingle, Fothergill, and Chandler (1938) stated that guinea pigs immunized with *Hemophilus influenzae* showed no circulating antibodies although they were fully protected. Futhermore, this protective action cannot be ascribed to the antitoxin because the rabbits immunized with rough organisms or their fractions were equally protected against smooth organisms presumably possessing the toxic somatic antigen. On the other hand, the presence of agglutinins and precipitins are not necessarily a proof that animals are protected. Immunization of rabbits with ketene-detoxified smooth organisms and their sonic lysates afforded no protection to the injected animals although their sera possessed these antibodies. The lack of protective antibodies in rabbits immunized with ketene-detoxified antigen suggests that ketene destroys not only the toxicity but also the unknown factor or factors indispensable for inducing protection.

A significant observation, which may have a bearing upon the nature of vaccine to be employed for human immunization, is that a nontoxic variant of *S. dysenteriae* afforded just as good qualitative and quantitative protection to the injected rabbits as did the toxic parent strain. It must, therefore, be concluded that toxicity of the antigen is not a determining factor in inducing immunity. However, the immunity afforded by heat-killed organisms and toxic products of *S. dysenteriae* seems not to be a lasting one.

SUMMARY

Immunization of rabbits with heat-killed organisms of smooth and rough variants of *Shigella dysenteriae* or some of its fractions affords protection against both of the variants and their toxic products.

The presence of agglutinins and precipitins in the sera of injected rabbits is not an index of protection.

The toxicity of the *S. dysenteriae* organisms is not a necessary adjunct of a protective vaccine.

The immunity afforded by the injection of a heat-killed vaccine of *S. dysenteriae* is of short duration.

The results of active protection experiments support the theory of one dominant antigen in *S. dysenteriae*.

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VITAMIN REQUIREMENTS OF THE GENUS PROPIONIBACTERIUM

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The literature concerning the nutrition of the propionic acid bacteria was reviewed by Thompson (1943). He attempted to determine which of the *Propionibacterium* species could be grown on synthetic media containing B vitamins and, if growth occurred, to show which vitamins were required. He was able to grow satisfactorily through repeated subculture five out of nine cultures. All five of these cultures capable of growth after repeated transfer required pantothenic acid, and four required biotin. Thiamine and *p*-aminobenzoic acid (PAB) appeared to be necessary or at least stimulatory in some cases.

Preliminary investigations in our laboratory concerning the nutritional characteristics of a few species, undertaken in the course of studies concerning the mechanism of propionic acid formation, indicated that under conditions of large inoculum the nutritional demands of these few cultures were very simple insofar as ability to undergo repeated transfer was concerned. Further studies revealed that with 25 different cultures representing 9 different species the only absolutely required B vitamins were pantothenic acid and biotin, with a few cultures requiring either PAB or thiamine for growth beyond the fifth or sixth serial transfer. Pantothenic acid alone was found to suffice for 3 of the 25 cultures. With all cultures growth was obtained through 15 serial transfers on media of known B vitamin content without loss in abundance or rapidity of growth, thus eliminating the possibility of transfer of growth factors through the inoculum.

METHODS

The basal synthetic medium had the following composition:

Glucose	10	g	$MgSO_4 \cdot 7H_2O$	160	mg
Casein hydrolyzate	5	"	$FeSO_4 \cdot 7H_2O$	8	"
Sodium acetate	8	"	$MnSO_4 \cdot 4H_2O$	3	"
K_2HPO_4	4	"	Adenine	5	"
Sodium thioglycolate	0.1	"	Guanine	5	"
L-Cystine	0.1	"	Uracil	5	"
L-Tryptophan	0.1	"	Xanthine	5	"
NaCl	4	"	H_2O to make one liter		

Adjusted to pH 6.8 to 7.0

Concentrated stock solutions of B vitamins were added to the basal medium in the preparation of the test media to give a concentration of 1 μg per ml of medium when pantothenic acid, PAB, and thiamine were added, and 1 mμg per ml when biotin was added. Incubation was at 30 C after a one-drop inoculation with an ordinary transfer pipette into 5 ml of test medium. Transfers were

made after 5 to 6 days of incubation. Negative tubes were held for at least 10 days before being discarded. Turbidity was measured directly from the culture tubes by means of an Evelyn type of photoelectric colorimeter.

TABLE 1

Summary of B vitamin requirements of Propionibacterium species

CULTURE	NO.	PANTOTHENIC ACID	BIOTIN	THIAMINE	PAB
P. shermanii	1	+	+	S	−
" "	2	+	+	S	−
" "	6	+	+	+	−
" "	7	+	+	S	−
" "	8	+	+	S	−
" "	9	+	+	−	−
" "	10	+	+	−	−
P. freudenreichii	13	+	+	−	−
" "	22	+	−	−	−
" "	23	+	−	−	−
" "	24	+	−	−	−
P. rubrum	1	+	+	S	+
" "	19	+	+	S	S
" "	9611	+	+	S	+
P. thoenii	20	+	+	+	−
" "	21	+	+	S	−
" "	25	+	+	+	−
P. jensenii	15	+	+	S	+
" "	16	+	+	S	−
" "	17	+	+	S	S
P. pentosaceum	14	+	+	S	−
" "	214	+	+	S	−
P. peterssonii	18	+	+	S	+
P. zeae	26	+	+	S	S
P. arabinosum	12	+	+	S	−

+, requiring; −, not requiring; S, not requiring but stimulating.

RESULTS

Under the conditions described, all cultures required pantothenic acid, and all but three required biotin (see table 1). Thiamine and PAB, as reported by Thompson (1943) and previous workers, were variable in their effect. In a few cases one or the other was seen to be required, at least for transfers beyond the

fifth or sixth. In many instances they stimulated but apparently were not required. Thiamine in particular was seen to enhance growth as much as twofold in a few cases, with varying degrees of stimulation being noted in the cases in which it is reported as stimulatory (table 1). The inclusion of pantothenic acid, biotin, PAB, and thiamine in the basal synthetic medium gave cultures which after the fifth or sixth serial transfer were as turbid as those obtained when the vitamins were replaced by yeast extract in a concentration of 1 mg per 10 ml of basal medium.

When amino acids were substituted for the casein hydrolyzate, continual serial transfer was possible, although in a few cases the extent of growth was considerably diminished.

<div align="center">DISCUSSION</div>

The general phenomenon of "training" of the *Propionibacterium* species to growth in the absence of a particular growth factor observed by Silverman and Werkman (1939) in studies with *P. pentosaceum* and its thiamine requirements, and also by Thompson (1943) in respect to the irregular response of *P. thoenii* to PAB, was likewise observed in these data; and, as suggested by Thompson (1943) concerning his investigations, supports the earlier observations by Wood *et al.* (1937, 1938) that these organisms have remarkable adaptive capacities. The latter workers also made the interesting and pertinent observation that certain strains showed "training" occurring more readily when a large inoculum was used. This phenomenon of "training" or "adaptation" can be considered as an experimental fact. It is the mechanism, of course, that remains unexplained. It was observed that in the earlier transfers from the complex initial medium containing yeast extract, growth response increased both in rapidity and extent as the transfer progressed. In one experiment, cultures after 15 transfers on synthetic media were found to give decidedly more rapid growth upon the next transfer as compared to the same strains just transferred from complex medium.

Although it could be argued that the large inoculum technique gives a poising advantage to generally anaerobic organisms, and that all cultures could be grown successfully because of this advantage, such an argument was not supported by experiments in which the addition of reducing substances such as cystine and sodium thioglycolate gave no noticeable increase in growth response when normal inoculation methods were used. The data would more generally support a theory of selection of the less demanding of the cells present in a heterogeneous population. An investigation of the possibilities involved in such a theory could well be made the basis of a separate study. In the opinion of the author, the failure of previous workers to cultivate successfully all of their cultures on synthetic media of known B vitamin content (when the B vitamins shown here were included) can be explained by the lack of sufficient numbers of "adapted" or "adaptable" organisms in the inoculum used. It can also explain the apparent lack of pattern as concerns the variable and sometimes irregular need for PAB and thiamine in the genus as a whole.

ACKNOWLEDGMENT

SUMMARY

By means of large inoculation, 25 different strains of the genus *Propionibacterium*, representing 9 different species, were successfully grown through repeated serial transfer on synthetic media. The B vitamin requirements were found to be relatively simple. The most demanding cultures required only pantothenic acid, biotin, and either thiamine or PAB. The majority of cultures required only pantothenic acid and biotin.

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THE VARIATION OF ANTIGENIC PATTERN AND OF MOUSE VIRULENCE IN AN INFLUENZA VIRUS CULTURE[1]

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Little is known of the changes, other than the obvious increase in mouse virulence, that occur in an influenza virus culture during the process of mouse adaptation. But recent studies by Hirst (1947) indicate that this process may be accompanied by a significant shift in antigenic pattern. Hirst reported two examples in which influenza virus isolated from throat washings by inoculation in eggs was antigenically different from, although related to, the virus isolated from the same throat washings by inoculation in ferrets, followed by mouse passage. The fact that the two egg-adapted strains were antigenically alike whereas the mouse-adapted strains not only differed from their egg counterparts but also differed from each other is good evidence that the antigenic differences resulted from changes that had occurred in the virus cultures during ferret-mouse passage.

Other reports indicate that an influenza virus may acquire mouse virulence without undergoing detectable antigenic change. Wang (1948) stated that the mouse-adapted line and the egg line (avirulent for mice) of the Rhodes strain of influenza virus were antigenically alike. Also, Anderson and Burnet (1947) found, during the adaptation of the egg-isolated Cam strain of virus to mice, that the virus, after it had acquired sufficient mouse virulence to produce lung lesions regularly and death frequently, was antigenically identical with the original Cam.

The present paper reports the results of antigenic studies that were made on an influenza virus culture at different stages of mouse adaptation. The data will show that there was a pronounced antigenic difference between the mouse-adapted and the egg-adapted lines of the virus, but that there was a lack of correlation between the change in antigenic pattern and the change in mouse virulence.

MATERIALS AND METHODS

Virus. The Cam strain (Anderson and Burnet, 1947) of influenza A prime virus was used in the present study. The virus had been isolated in embryonated eggs and was received in this laboratory as frozen and dried allantoic fluid from the twenty-second egg passage. In this report Cam E is used to indicate the virus that had been cultivated exclusively in the chick embryo and that was in the sixty-first egg passage at the beginning of the study; passage was routinely

made by intra-allantoic inoculation of a 10^{-3} dilution of infected allantoic fluid. Cam M refers to the virus that had been through at least 30 mouse passages and that possessed a relatively high degree of virulence for that species. This line was started with allantoic fluid from the thirty-eighth egg passage and subsequent passages were made with 10 per cent lung suspensions at 3- or 4-day intervals. Cam E-M indicates the virus present in the lungs of second passage mice; as shown previously (Sugg, 1949), the virus at that stage of adaptation can multiply to a high egg-infectious titer in the mouse lung but does not possess sufficient virulence to produce lesions when mouse to mouse passage is made.

For both the neutralization and the agglutination inhibition tests infected allantoic fluids were used as antigens; in the case of Cam M and Cam E-M the fluids were obtained from eggs that had been inoculated with infected mouse lungs.

TABLE 1

Demonstration of antigenic differences between the egg line (E) and the mouse-adapted line
(M) of the Cam virus by means of cross-neutralization tests in eggs

ANTISERUM			TEST VIRUS	
Virus used for immunization	Animal*	Number of inoculations	Cam E	Cam M
Cam E	Rabbit 39	2	$3^{-6.3}$†	$3^{-4.0}$
(allantoic fluid)	Rabbit 39	4	$3^{-7.5}$	$3^{-6.7}$
	Mouse pool	2	$3^{-5.5}$	$3^{-3.6}$
Cam M	Rabbit 40	2	$3^{-4.6}$·	$3^{-6.8}$
(mouse lung)	Rabbit 40	4	$3^{-5.5}$	$3^{-7.4}$
	Mouse pool	2	$3^{-3.5}$	$3^{-6.6}$

* All of the animals were immunized by the intraperitoneal route; the rabbits received 10 ml and the mice received 0.5 ml at each inoculation.

† The calculated initial dilution of serum that protected 50 per cent of the eggs from infection.

Antiserums. Immunization was conducted either with undiluted virus-containing allantoic fluids or with 10 per cent suspensions of infected mouse lungs; all of the preparations had a high titer of egg-infectious virus. Serum was obtained from each animal 14 days after the last inoculation. The details of the various immunization procedures are shown in tables 1 and 2.

Neutralization tests. Embryonated eggs were used for these tests and the procedure was essentially the same as that described by Hirst (1942). A number of tubes of each of the virus-containing allantoic fluids, which were used as antigens, were frozen in the CO_2 ice chest, and, as required, a tube was removed and diluted to the desired concentration with sterile infusion broth; the test dose contained between 100 and 1,000 egg-infectious doses of virus.

The antiserums were inactivated at 56 C for 30 minutes, after which a series of threefold dilutions were prepared in saline. To each serum dilution was added an equal volume of virus suspension and sufficient penicillin to provide 100

units per inoculated egg. The contents of the tubes were thoroughly mixed and incubated at 37 C for 30 minutes. Two-tenths ml of each mixture was then inoculated into the allantoic sac of each of three 11-day-old chick embryos. After 65 hours' incubation at 35 C, allantoic fluid was obtained from each egg and tested for the presence of hemagglutinins; in the absence of agglutination the egg was considered free of infection. The titers are expressed as the calculated initial dilution of serum that protected 50 per cent of the eggs from infection (Reed and Muench, 1938). Most of the serums were included in two, and many in three, separate tests with the same antigen, and the results were combined in

TABLE 2

Results of cross tests between different lines of the Cam virus and their antiserums

ANTISERUM			TITER* OF DIFFERENT LINES OF VIRUS					
			Neutralization			Agglutination inhibition		
Virus used for immunization	Material inoculated†	Animal	Cam E	Cam E-M	Cam M	Cam E	Cam E-M	Cam M
Cam E	Al. Fl.	Ferret 1	$3^{-6 \cdot 3}$	$3^{-6 \cdot 2}$	$3^{-4 \cdot 3}$	640	640	80
	" "	" 2	$3^{-5 \cdot 5}$	$3^{-5 \cdot 5}$	$3^{-4 \cdot 1}$	320	320	80
	" "	Rabbit 44	$3^{-3 \cdot 5}$	$3^{-3 \cdot 5}$	$3^{-1 \cdot 6}$	40	40	<20
	" "	" 45	$3^{-2 \cdot 7}$	$3^{-2 \cdot 5}$	$3^{-1 \cdot 5}$	40	40	<20
Cam E-M	M. L.	Ferret 3	$3^{-6 \cdot 4}$	$3^{-5 \cdot 6}$	$3^{-4 \cdot 5}$	640	640	80
	"	" 4	$3^{-4 \cdot 7}$	$3^{-4 \cdot 5}$	$3^{-4 \cdot 2}$	320	160	40
	Al. Fl.	Rabbit 46	$3^{-4 \cdot 6}$	$3^{-4 \cdot 5}$	$3^{-3 \cdot 5}$	80	80	40
	" "	" 47	$3^{-5 \cdot 5}$	$3^{-5 \cdot 3}$	$3^{-2 \cdot 2}$	160	160	20
Cam M	M. L.	Ferret 5	$3^{-2 \cdot 6}$	$3^{-3 \cdot 2}$	$3^{-8 \cdot 7}$	40	40	1,280
	"	" 6	$3^{-2 \cdot 5}$	$3^{-2 \cdot 5}$	$3^{-8 \cdot 6}$	40	20	1,280
	Al. Fl.	Rabbit 48	$3^{-2 \cdot 0}$	$3^{-2 \cdot 0}$	$3^{-6 \cdot 5}$	20	20	160
	" "	" 49	$3^{-2 \cdot 8}$	$3^{-2 \cdot 5}$	$3^{-6 \cdot 5}$	40	40	320

 * The neutralization titer is the calculated initial dilution of serum that protected 50 per cent of the eggs from infection. The agglutination inhibition titer is the reciprocal of the highest initial dilution of serum that completely inhibited 6 units of agglutinin.

 † Each ferret received one intranasal inoculation of 1.5 ml and each rabbit received one intraperitoneal inoculation of 5 ml of either undiluted infected allantoic fluid (Al. Fl.) or 10 per cent suspension of infected mouse lung (M. L.).

calculating the serum titers. Embryos that were found dead 24 hours after inoculation were discarded. The final results therefore were based upon the use of 3 to 9 eggs for each serum dilution.

Agglutination inhibition tests. This procedure was conducted by a pattern test (Salk, 1944) in which equal volumes (0.02 ml) of virus suspension, serum dilution, and 0.5 per cent chicken erythrocytes were combined and incubated at room temperature until the cells had settled sufficiently (approximately 45 minutes) for the results to be determined. The serums were tested in twofold dilutions and the titers are expressed as the reciprocal of the highest initial dilution of serum that completely inhibited 6 units of agglutinin.

Comparison of the antigenic patterns of different lines of the Cam strain of virus.
Antiserums against the egg line (Cam E) and against the mouse line (Cam M) of
the Cam strain of virus were prepared by the intraperitoneal inoculation of
rabbits and of mice. Bleedings were made after two inoculations and, in the
case of the rabbits, additional bleedings were obtained after four inoculations.
All of the serums were then titrated against each virus line by means of egg neu-
tralization tests. It is evident from the results, which are shown in table 1, that
there was a pronounced difference in the neutralizing capacity of each serum
when it was tested against the two lines of virus. The greatest difference was
shown by the mouse serums; the Cam E antiserum had a titer of $3^{-5.5}$ against
the homologous virus but a titer of only $3^{-2.6}$ against the heterologous virus,
and the Cam M antiserum had corresponding titers of $3^{-6.6}$ and $3^{-2.5}$. Differ-
ences of less extent were shown by the rabbit serums, but they were of sufficient
magnitude to indicate a true antigenic difference between the two lines of virus,
particularly in view of the extensive immunization that those animals had
received.

The difference between the two lines suggested that the mouse lung might
have served as a "selective medium" that favored the growth of a variant that
differed in antigenic properties from most of the particles comprising the original
virus population. Information on that point was obtained from the following
experiment: Antiserums were prepared against the virus (Cam E-M) present
in the lungs of second passage mice by intranasal inoculation of ferrets and by
intraperitoneal inoculation of rabbits. At the same time and with the same im-
munization procedures, antiserums were prepared against Cam E and against
Cam M. All of the antiserums were then titrated against each of the three lines
of virus in both neutralization and agglutination inhibition tests. The results
are presented in table 2.

It will be seen that, with either method of test, the titer of each serum was
the same, or varied to only an insignificant extent, against Cam E and Cam E-M.
Since 12 serums, produced by various immunization procedures in two different
animal species, were included, these data show quite clearly that after two mouse
passages the virus (Cam E-M) was closely similar to, if not identical with, the
original egg-adapted virus (Cam E) in antigenic properties. It is evident, there-
fore, that the ability to grow in the mouse lung was independent of the change
in antigenic pattern. However, it should be emphasized that the virus at that
stage of adaptation (second mouse passage) had not acquired sufficient virulence
to produce lung lesions when passed to normal mice by the usual method.

The pronounced antigenic difference between Cam M on the one hand and
Cam E and Cam E-M on the other hand is again illustrated by the results
included in table 2. Each of the Cam M antiserums had a much higher titer,
and each of the Cam E and Cam E-M antiserums had a lower titer, when tested
against Cam M than when tested against either of the other two lines. Inspec-
tion of the titers also will show that immunization with Cam M resulted in
antiserums with lower ratios of heterologous to homologous antibodies than did

immunization with either of the other viruses. This difference was especially marked when comparison was limited to the ferret serums. For example, ferret 5 had a neutralization titer ratio of $3^{-3.2}$ to $3^{-8.7}$, which was the highest shown by either of the Cam M ferret serums, whereas ferret 1 had a ratio of $3^{-3.4}$ to $3^{-6.3}$, which was the lowest found with any of the Cam E or Cam E-M ferret serums. Although only a small number of animals were included in the experiment, these results indicate that there was a fundamental difference in the antibody-evoking capacities of the different lines of virus.

Lack of correlation between change in mouse virulence and change in antigenic pattern. The following experiment compares the virus at different stages of mouse adaptation in respect to mouse virulence and to antigenic pattern. Allantoic fluid from the ninety-third egg passage was used as starting material, and

TABLE 3

Lack of correlation between change in mouse virulence and change in antigenic pattern

VIRUS FROM MOUSE PASSAGE NO.[*]	MOUSE VIRULENCE (INTRANASAL TITRATION)		ANTIGENIC PATTERN (AGGLUTINATION INHIBITION TITER OF DIFFERENT ANTISERUMS)		
	Mortality	Lesions	F-1	F-3	F-5
0	$10^{-0.5}$	$10^{-0.5}$	640	640	80
1	0†	0†	320	640	80
2	0	0	320	640	40
3	0	0	320	320	40
4	0	$10^{-1.5}$	320	640	40
5	$10^{-1.5}$	$10^{-2.5}$	320	320	40
6	$10^{-2.5}$	$10^{-4.5}$	320	320	40
7	$10^{-3.3}$	$10^{-5.5}$	320	320	40
8	$10^{-4.2}$	$10^{-6.5}$	320	320	40
60	$10^{-5.3}$	$10^{-6.8}$	80	80	1,280

* Passage no. 0 indicates the allantoic fluid starting material, which was from the 93rd egg passage. Passage no. 60 was the Cam M virus that had been started with allantoic fluid from the 38th egg passage.

† 0 indicates either that there were no deaths or that there were no lung lesions in mice that had received 10 per cent lung suspension, which was the highest concentration tested.

passage was made at 3- or 4-day intervals by the intranasal inoculation of 10 per cent lung suspension. Each of the passage materials, and for comparison a like preparation from the sixtieth mouse passage of Cam M, was inoculated into the allantoic sac of 10- or 11-day-old chick embryos and, at the same time, was titrated in mice. The mice were observed for deaths, and at the end of 10 days survivors were sacrificed and examined for lung lesions. The inoculated eggs were incubated for from 48 to 72 hours at 35 C when allantoic fluids were harvested and stored at 4 C until the end of the experiment. The fluids were then compared in an agglutination inhibition test in which 3 antiserums, each produced by immunization with a different line of virus, were titrated against 6 agglutinating units of each virus preparation. The results of that experiment are shown in table 3.

For proper interpretation of the data in table 3, it should be pointed out that the difference between the mortality titer of the allantoic fluid used for passage no. 0 ($10^{-0.5}$) and the titers of the lung suspensions used for the three subsequent passages (0) does not indicate a decrease in virulence on the part of the virus, but is a function of the concentration of active virus contained in the inoculum.

It is evident (table 3) that the virus showed a marked increase in mouse virulence without any detected change in antigenic pattern. Beginning with passage no. 4, which had a mortality titer of 0 and a lesion titer of $10^{-1.5}$, there was a consistent increase in virulence so that at passage no. 8 the corresponding titers were $10^{-4.3}$ and $10^{-6.5}$. At that stage of adaptation (passage no. 8) the virulence was only slightly less than was found for Cam M, which had been through 60 mouse passages and which had mortality and lesion titers of $10^{-5.3}$ and $10^{-6.8}$, respectively. In contrast, during the first 8 passages, no significant antigenic change was apparent, as determined by the agglutination inhibition tests: the titers of the Cam E (F-1) and Cam E-M (F-3) antiserums were 320 to 640 and the titer of the Cam M (F-5) antiserum was 40 to 80 against all of those virus preparations. There was, however, a marked antigenic difference

TABLE 4

Antigenic similarity of the virus before and after eight mouse passages as shown by egg neutralization tests

TEST VIRUS	ANTISERUM		
	F-1	F-3	F-5
Before mouse passage (Cam E).............	$3^{-6.3}$	$3^{-6.2}$	$3^{-2.6}$
After 8 mouse passages	$3^{-6.5}$	$3^{-6.5}$	$3^{-2.7}$

between those preparations and the Cam M virus; the F-1 and F-3 serums had a titer of 80 and the F-5 serum had a titer of 1,280 against the latter virus.

The antigenic similarity of the virus present in the lungs of the eighth passage mice to the original Cam E virus was confirmed by neutralization tests. The results of those tests are shown in table 4.

Thus, when compared on the basis of antigenic pattern, the virus from passage no. 8 was closely similar to Cam E and widely different from Cam M, but, when compared on the basis of mouse virulence, the relationship was reversed. It would appear, therefore, that variation in either one of those properties may occur independently of any significant change in the other.

DISCUSSION

The recorded experiments provide ample evidence of a true antigenic difference between a line of the Cam strain of influenza virus that had been propa-. gated for more than 30 transfers in mouse lung and the original line of that virus that had been maintained in chick embryos. The mouse line was started after the virus had been through 38 egg passages and when it was sufficiently adapted to that host to multiply to high titer following intra-allantoic inoculation.

Hence, the observed difference between the two lines furnish an example of antigenic variation in an established laboratory strain of influenza virus. In that respect the present study differed from that of Hirst (1947) in which both the egg and the mouse lines were obtained directly from the material from the patient (throat washings) by different isolation procedures. Evidence of antigenic variation in a laboratory strain of influenza virus also has been reported by Francis (1947); in that instance the variation occurred in a line of the mouse-adapted PR8 strain that had been maintained in tissue cultures and in eggs. All of these observations indicate that the influenza viruses are relatively unstable agents that may show shifts in antigenic pattern with adaptation to new host species.

The present data also show (table 3) that a third line of the Cam virus that had been through 8 mouse passages and that was highly virulent for the host was closely similar to, if not identical with, the original egg line in antigenic properties. The 8 passages may not have produced a fully mouse-adapted virus and it is possible that antigenic change might have occurred with additional passage. But sufficient passages were made to show that a virus that was relatively avirulent for mice was converted into one that was highly virulent for that species without any apparent shift in its antigenic pattern. These results are of interest as an indication that the antigenic changes that may occur during the adaptation of an influenza virus to mice are unrelated to the ability of the virus to survive in the lung tissue or to acquire virulence for the host.

In interpreting the results of all studies on variation in influenza viruses, one must take into account the fact that there is no adequate method available, analogous to the plating of bacteria, whereby cultures can be derived from a single virus particle. Therefore, however remote, the possibility must be considered that differences between two lines of a strain of influenza virus may be the result of a selection of different viruses present in the starting material rather than the result of variation in an originally homogeneous virus population.

SUMMARY

A marked antigenic difference was found to exist between a line of the Cam strain of influenza A prime virus that had been through 30 or more mouse passages and the original line of that same strain that had been maintained in chick embryos. The data show that, although antigenic change may occur with mouse adaptation, an influenza virus that is relatively avirulent for mice may be converted into one that is highly virulent for that species without any apparent change in its antigenic pattern.

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NOTE

THE OCCURRENCE OF VEGETATIVE CELLS OF CLOSTRIDIUM PERFRINGENS IN SOIL

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A considerable difference of opinion exists as to the form in which the pathogenic clostridia, and particularly *Clostridium perfringens*, exist in the soil. Some authors consider that the soil is a natural habitat of this species. Others believe that *C. perfringens* leads a saprophytic life in the intestinal contents of man and other animals, that the organisms are excreted with feces, and that only spores resulting from fecal contamination are to be found in the soil.

In order to obtain information on this point the relative numbers of heat-susceptible and heat-resistant forms were determined in eight samples of soil that had not been exposed to known fecal contamination for some years. Duplicate portions of each soil sample were diluted 10^1 in sterile M/60 phosphate buffer (pH 7.0). One of each pair of 10^1 dilutions was heated for 20 minutes at 78 C. Serial dilutions in phosphate buffer of 10^2, 10^3, 10^4, and 10^5 were then made from the unheated and heated suspensions. From each dilution 10 tubes of chopped meat medium (pH 7.4) containing 1 per cent glucose were inoculated with 0.5 ml and were incubated for 8 to 12 hours at 37 C. From each chopped meat tube a tube of whole milk medium was inoculated, and the milk tubes were incubated for 12 to 18 hours. From each milk tube showing production of acid and gas, a blood agar plate was inoculated. After anaerobic incubation of these plates, colonies resembling those of *C. perfringens* were transferred to semisolid medium for the determination of motility and morphological characteristics. The ability of a number of the strains to ferment glucose, lactose, sucrose, and salicin was determined. The "most probable number" of organisms per gram of soil (on a dry weight basis) for each sample was calculated by the method of Halvorson and Ziegler (*Quantitative Bacteriology*, Burgess Publishing Co., Minneapolis, 1938), with 10 tubes for each dilution as is indicated above. The results are shown in table 1.

It should be pointed out that the numbers of organisms presented in this table are based on actual isolations. The true numbers of *C. perfringens* in the various samples may be larger, because of inadequacies of the technique used for isolation, but they are quite unlikely to be smaller. Comparative data on heat-susceptible forms of *C. perfringens* were not obtainable for one sample of soil because it contained such large numbers of an anaerobic micrococcus, which produced "stormy fermentation" of milk, that *C. perfringens* could not be isolated on a quantitative basis.

The data in table 1 indicate that large numbers of *C. perfringens* can exist in the soil in heat-susceptible form. Because of the relatively high heat resistance

TABLE 1

Numbers of C. perfringens per gram (dry weight) of soil

ORIGIN OF SOIL SAMPLE	pH OF SOIL	HEAT-RESIS-TANT	TOTAL
Clayey loam from uncultivated field....	5.4	790	1,090
Black loam from creek bank	5.3	5,940	56,700
Sandy garden loam...............................	. 6.0	703	1,730
Clayey garden soil............................ ..	5.7	73	110
Muck from swamp...............	4.5	464	1,530
Sandy soil and decaying vegetation........	6.0	200	1,150
Soil underlying turf.................	6.3	70	1,200

of *C. perfringens* spores reported by Weinberg, Nativelle, and Prévot (*Les Microbes anaerobies*, Masson et Cie., Paris, 1937), it appears that the heat-susceptible forms are probably vegetative cells. It follows that multiplication of this species takes place in the soil, and, consequently, that soil is a natural habitat of this species and that it is not necessary to postulate fecal contamination to account for its presence in soil.

RHODOMICROBIUM VANNIELII, A NEW PHOTOHETEROTROPHIC BACTERIUM

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Studies of the photoheterotrophic, or nonsulfur purple, bacteria have shown that these microorganisms are closely related morphologically, and can be placed either in the genus *Rhodospirillum* or *Rhodopseudomonas* (van Niel, 1944). These two genera comprise the family *Athiorhodaceae*. In view of the morphological homogeneity of the nonsulfur purple bacteria, it was, therefore, of considerable interest when one of a number of enrichment cultures prepared for organisms of this family supported the development of a photoheterotrophic bacterium in which the morphology and mode of cell division differed markedly not only from members of the *Athiorhodaceae* but from other *Schizomycetes* as well. Subsequent studies of the morphology and physiology of several pure cultures have led us to create a new genus, *Rhodomicrobium*, for these organisms. The type species of the new genus is *R. vannielii*. We have chosen this species name in honor of Professor C. B. van Niel, whose studies of the photosynthetic bacteria have added so much to our knowledge of this group of microorganisms. Definitions of the new genus and species are given in a later section of this paper.

METHODS

The original culture was obtained following the inoculation of mud into the medium suggested by van Niel (1944), which consists of NaHCO$_3$, 0.5 per cent; NaCl, 0.2 per cent; (NH$_4$)$_2$SO$_4$, 0.1 per cent; K$_2$HPO$_4$, 0.05 per cent; MgSO$_4$·7H$_2$O, 0.01 per cent; Na$_2$S·9H$_2$O, 0.01 per cent; organic hydrogen donor (ethanol in the case above), 0.2 per cent; pH, 7.0. Glass-stoppered bottles were completely filled with the inoculated medium and incubated under continuous illumination at 25 to 30 C. *R. vannielii* was the predominant organism in the culture after 7 days' incubation. Many similar enrichment cultures have subsequently been made employing a variety of organic donors and inocula from different sources. Although *Rhodomicrobium* has been seen a number of times in these cultures, it ordinarily has been so outnumbered by other nonsulfur purple bacteria that isolation was impossible. Nevertheless, one additional strain was obtained from such enrichment cultures. Two other strains have been isolated from enrichment cultures for *Thiorhodaceae* that contained 0.1 per cent Na$_2$S·9H$_2$O in place of an organic hydrogen donor. Growth of *Rhodomicrobium* in the sulfide-containing enrichment cultures was slow and very sparse, and occurred subsequent to the development of the sulfur purple bacteria.

The agar medium used for the shake cultures in isolation and purification was prepared according to the procedures suggested by van Niel (1944). The composition was the same as for the enrichment cultures except that 0.2 per cent by

volume of yeast autolyzate was added to stimulate growth. The medium, with the exception of the bicarbonate, sulfide, and ethanol, was autoclaved in Erlenmeyer flasks, and to it, after it had been cooled to 50 C, appropriate amounts of the foregoing three constituents were added aseptically from solutions that had been sterilized by pressure filtration. The pH was then adjusted to 6.8 to 7.2 with sterile 5 per cent phosphoric acid. The molten medium was dispensed into sterile soft-glass test tubes and immediately inoculated with dilutions of material from the enrichment cultures. Since these organisms are strict anaerobes, it was necessary to seal the shake tubes with a layer of sterile "vaspar" to obtain consistently successful cultures. The selection of colonies of R. vannielii in shake tubes seeded from a mixed population was facilitated by the deep red color and characteristic rough, convoluted surface of the colonies (figure 1). Isolated strains remained viable in stab cultures for at least six months.

Figure 1. Colonies of R. vannielii in shake culture. × 2.

MORPHOLOGY AND MODE OF CELL DIVISION

The distinguishing morphological features of R. vannielii are the attachment of the cells by means of a slender, branched filament and the mode of reproduction, which we believe to be by budding rather than fission. Figure 2 illustrates typical cell groups taken from a 7-day-old culture and photographed while suspended in dilute gentian violet. The individual cells at maturity are ovoid in shape with dimensions of approximately 1.2 by 2.8 microns. The connecting filaments vary greatly in length but are uniformly about 0.3 microns in diameter. The electron micrograph shown in figure 3 reveals that a short portion of the filament connecting two mature cells is generally constricted and considerably more opaque to the electron beam than the remainder of the filament.

It can be observed that many of the terminal cells of cell groups possess filaments that vary in length from very short protuberances to structures several microns long. The tips of the filaments may be undifferentiated (figure 6, left) or swollen (figure 6, right) to various degrees. We believe that these globose structures at the tips of the filaments are new cells in various stages of development. The size range of the terminal cells is well illustrated in figure 2. Cell multiplication appears to be initiated by the outgrowth of a new filament from the pole of a mature or immature cell, or from some point along a filament connect-

ing two cells (figures 7, 5). Following a period of elongation of the new filament, its tip enlarges to form a daughter cell. It appears that branching of the filaments is due almost exclusively to lateral outgrowths from the filaments con-

Figure 2. R. vanntelii Seven-day-old culture photographed in dilute gentian violet × 1,800.

Figure 3. R. vanntelii Seven-day-old culture showing constriction of the filament. Electron micrograph, × 10,000.

Figure 4. R. vanntelii. Thirteen-day-old culture showing bud arising from the tip of an immature cell. Electron micrograph, × 8,400.

Figure 5. R. vanntelii Seven-day-old culture showing terminal and lateral filaments. Electron micrograph, × 9,000.

necting the cells rather than to longitudinal fission of terminal cells, although what seemed to be bifurcation of an undifferentiated tip has been observed on one occasion (figure 10).
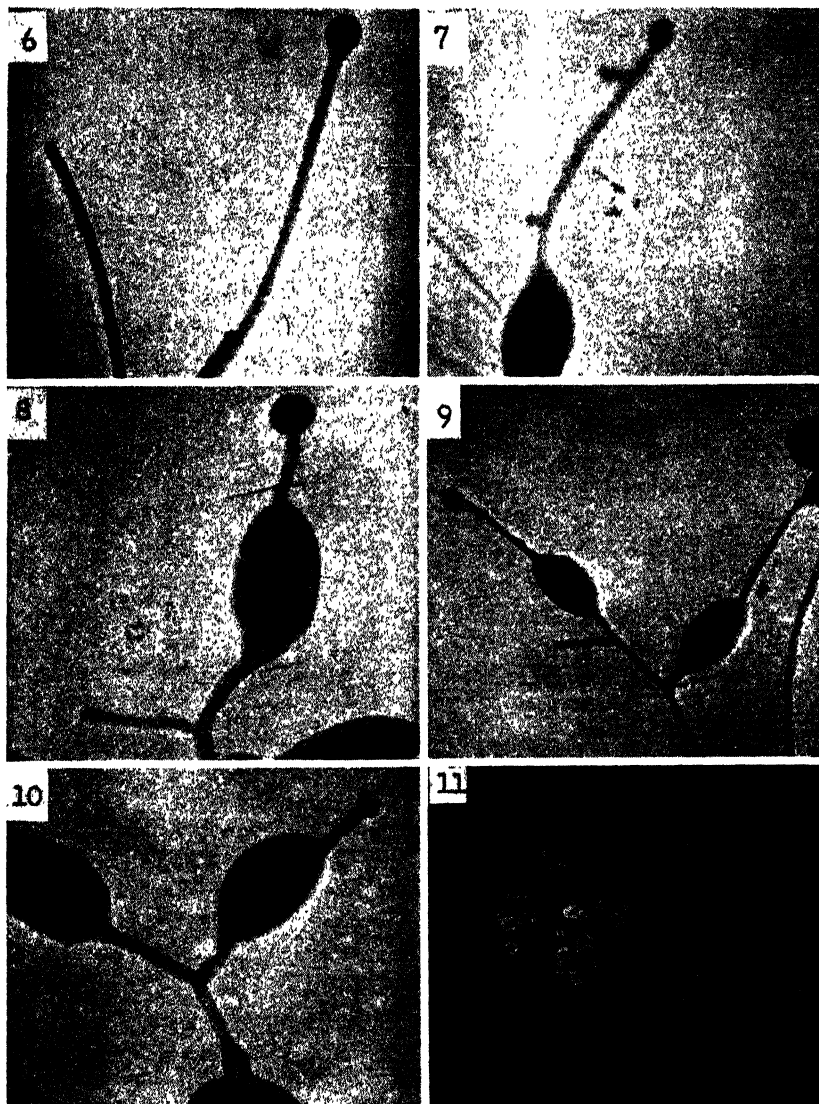
Figure 6. R. vannielii. Seven-day-old culture. On the left is a filament, the tip of which is undifferentiated. The tip of the filament on the right has swollen to form an immature cell. A broken filament can be seen lying alongside the filament on the right. Electron micrograph, × 8,400.

Figure 7. R. vannielii. Fourteen-day-old culture showing buds arising from the filament connecting a mature and an immature cell. Electron micrograph, × 8,400.

Figure 8. R. vannielii. Three-day-old culture. Electron micrograph, × 9,000.

Figure 9. R. vannielii. Three-day-old culture. Electron micrograph, × 5,600.

Figure 10. R. vannielii. Thirteen-day-old culture showing bifurcation of the tip of a filament. Electron micrograph, × 9,000.

Figure 11. Hypomicrobium vulgare. Dark-field photomicrograph, × 1,400. After Kingma-Boltjes.

Because of difficulties encountered in growing *Rhodomicrobium* in slide cultures, we have not yet made direct observations of the mode of growth. Nevertheless, our failure to find any evidence of cells dividing by either transverse or longitudinal fission in cultures of various ages, together with the general morphological features of the organism, makes it reasonable to believe that this bacterium multiplies in the manner described above. Since the process of budding is understood to involve the formation of a new cell from a protuberance of part of another cell, we believe that the use of the term budding to describe the mode of cell multiplication in *Rhodomicrobium* is justified, even though the process differs considerably from that in yeasts in which the bud develops directly into a daughter cell without a preceding elongation to form a filament.

The morphology of *Rhodomicrobium* is quite constant regardless of the type of organic donor present in the culture medium. This is in contrast to the pronounced effect of different hydrogen donors on the morphology of *Rhodopseudomonas* and *Rhodospirillum* (van Niel, 1944).

Neither resting stages nor motile forms have been observed, and the gram reaction is negative. Mature cells contain refractive globules which by staining with Sudan black B have been shown to be fat.

PHYSIOLOGY AND BIOCHEMICAL ACTIVITY

An organic hydrogen donor, carbon dioxide furnished as bicarbonate, and light are required for growth. The organisms are obligately photosynthetic and obligately anaerobic. No growth has ever been obtained except in strictly anaerobic illuminated cultures. Preliminary investigations of the pigment system of one strain have demonstrated the presence of bacteriochlorophyll and a number of carotenoids (Volk and Pennington, 1949).

Growth factors are not required. About half-maximal growth is obtained in media containing ethanol as the only organic compound, and serial transfers in this medium have shown no diminution of growth. Growth in ethanol medium is stimulated by small amounts of yeast autolyzate, although the amount of growth obtained at the expense of the yeast autolyzate in the absence of ethanol is negligible (table 1). Yeast autolyzate as a growth stimulant could not be replaced by mixtures of B vitamins or amino acids. When the vitamin and amino acid supplements used by Henderson and Snell (1948) for the cultivation of lactic acid bacteria were added either separately or together to otherwise unsupplemented medium, no stimulation of growth was effected. In fact, an amino acid concentration of 0.01 per cent inhibited growth markedly, and at 0.05 per cent amino acid concentration inhibition was complete. The ability of *R. vannielii* to grow without an exogenous supply of growth factors is an important difference between the nutritional requirements of this organism and members of the *Athiorhodaceae*, for none of the latter organisms will grow in unsupplemented medium (van Niel, 1944; Hutner, 1946).

Hydrogen donors that give equally good cultures at 0.2 per cent concentration are ethanol, propanol, butanol, acetate, propionate, butyrate, valerate, caproate, and lactate. Malate is utilized slowly, but glucose, mannose, fructose,

sorbitol, mannitol, citrate, tartrate, formate, thiosulfate, and sulfide are not utilized. Since two of our four strains were isolated from enrichment cultures containing 0.1 per cent sulfide in place of an organic donor, the ability of pure cultures to use this donor has been tested a number of times at several different sulfide concentrations. However, we have been unable to obtain any evidence that sulfide is utilized at a significant rate (table 2). It must be concluded, therefore, that growth of *Rhodomicrobium* in the sulfide-containing enrichment cultures occurred at the expense of small amounts of organic matter present in

TABLE 1

Effect of yeast autolyzate on growth of R. vannielii in media with and without ethanol

(The medium contained inorganic salts plus the additions indicated in the table. Incubation period, 7 days at 28 to 30 C)

% BY VOLUME YEAST AUTOLYZATE	OPTICAL DENSITY	
	0.2% Ethanol	No ethanol
0	0.315	0
0.01	0.325	0.006
0.05	0.440	0.035
0.1	0.380	0.075
0.2	0.620	0.017
0.5	0.555	0.025

TABLE 2

Effect of sulfide on growth of R. vannielii in media with and without ethanol

(The basal medium contained inorganic salts plus 0.2 per cent by volume yeast autolyzate. Final pH 7.3; incubation, 7 days at 29 to 30 C)

$Na_2S \cdot 9H_2O$, %	OPTICAL DENSITY	
	0.2% Ethanol	No ethanol
0.01	0.72	0.038
0.02	0.75	0.041
0.04	0.67	0.044
0.06	0.76	0.047
0.08	0.49	0.035
0.10	0.58	0.032

the inoculum or elaborated by the sulfur purple bacteria that developed first in such cultures.

TAXONOMIC POSITION AND POSSIBLE RELATIONSHIP TO HYPHO-MICROBIUM VULGARE

Although *Rhodomicrobium* is closely related biochemically to the nonsulfur purple bacteria, its morphology and mode of cell division preclude its inclusion in the *Eubacteriales*, let alone the *Athiorhodaceae*. Since there is no other order of the *Schizomycetes* in which *Rhodomicrobium* may be placed, we believe it best to

include this organism in a provisional appendix to the *Schizomycetes* until more
is known concerning the existence of other bacteria that may possess a similar
mode of cell division. The inclusion of microorganisms of unknown relation-
ships in an appendix to the *Schizomycetes* has been recommended by Stanier
and van Niel (1941) on the ground that such a procedure stimulates further
investigation of the organisms therein because of the tentative nature of such
a treatment.

We believe there is some evidence to indicate a possible relationship between
Rhodomicrobium and *Hyphomicrobium vulgare*, the chemoheterotrophic bacte-
rium commonly found in enrichment cultures for nitrifying bacteria.

Hyphomicrobium has been described (Rullman, 1897, 1898; Stutzer and Hart-
leb, 1899) as a small, rod-shaped or egg-shaped bacterium that produces small
threads which may be branched. Stutzer and Hartleb considered the threads
as mycelial in nature and the bacterial cells as chlamydospores from which the
mycelium sprouts. Henrici and Johnson (1935), although they made no observa-
tion of *Hyphomicrobium* themselves, took an entirely different view of the na-
ture of the filamentous structures of this bacterium since they considered them
to be analogous to the lifeless stalks of the stalked bacteria. This interpretation
was accepted by Stanier and van Niel (1941). The only recent studies of this
organism have been made by Kingma-Boltjes (1934, 1936), who was unable to
come to any conclusion concerning the significance of the filaments or the
mechanism of cell division. He expressed the opinion, however, that cell division
was probably different from that of other bacteria. Kingma-Boltjes' photo-
micrographs of *Hyphomicrobium*, one of which is reproduced in figure 11, clearly
show the extensive branching of the filaments and the egg-shaped cells that
occur at the tips of the filaments. These morphological features suggest to us
that the filaments of *Hyphomicrobium* are not stalks in the sense that this term
has been used by Henrici to describe the nonprotoplasmic attaching structures
that are secreted by the true stalked bacteria, but are instead living structures
analogous in function to the filaments found in *Rhodomicrobium*.

Definition of the Genus and Species

Rhodomicrobium, nov. gen. Oval to round bacteria, attached by means of a
slender branched filament. Cell multiplication is initiated by the outgrowth of
a new filament from the pole of a mature or immature cell, or from some point
along a filament connecting two cells. The tip of the filament swells to form a
round cell, which increases in size and eventually assumes an ovoid shape.
Nonmotile, nonsporeforming, gram-negative. Contain bacteriochlorophyll,
which enables them to have a photosynthetic metabolism dependent on extrane-
ous oxidizable compounds and not accompanied by oxygen production. Contain
carotenoid pigments, which give cultures a salmon-pink to a deep orange-red
color, depending on the density of growth. The type species is *R. vannielii*.

Rhodomicrobium vannielii, n. sp. Morphology and mode of cell division as
described above. Mature cells are ovoid, 1.2 by 2.8 microns. The filaments are
approximately 0.3 microns in diameter. Growth occurs only in illuminated

anaerobic cultures and at the expense of organic hydrogen donors. Sulfide, thiosulfate, and sugars not utilized; organic growth factors not required. Gelatin not liquefied. Catalase-positive. Growth in fluid cultures flocculent, the color varying from a salmon pink to a deep orange red. Colonies are dark red and irregular in shape, and have a rough, convoluted surface.

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SUMMARY

Rhodomicrobium vannielii, a new photoheterotrophic bacterium, has been described. The distinguishing morphological features of this microorganism are the attachment of the cells by means of a branched filament and the mode of reproduction, which we believe to be by budding.

The possibility of a relationship between *Rhodomicrobium* and *Hyphomicrobium vulgare* is discussed.

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THE EXTRA OXYGEN CONSUMED DURING GROWTH OF SERRATIA MARCESCENS AS A FUNCTION OF THE CARBON AND NITROGEN SOURCES AND OF TEMPERATURE[1]

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It has recently been shown that *Escherichia coli* (Armstrong and Fisher, 1947) and *Serratia marcescens* (McLean and Fisher, 1947) consume oxygen more rapidly during a period of active growth than when the cells are in a resting condition. For these experiments the bacteria were maintained and examined in a medium containing only salts with citrate or glycerol as the carbon source and with ammonia as the sole source of nitrogen. It was observed that the rate of oxygen consumption of cells which had been growing in a respirometer vessel fell, following the exhaustion of the ammonia, to a level that was approximately 60 per cent of that in existence at the moment the uptake of ammonia was completed. The implication is that the growth process requires oxidative energy at a rate higher than that at which oxidative energy is liberated in the resting cell. Before drawing this general conclusion, however, it is necessary to know that the result observed is independent of the nitrogen and carbon sources employed, and that it is not a peculiarity seen only in the particular medium used heretofore. We have therefore repeated this type of experiment with several other nitrogen sources and with several carbon sources; and as a further test of the variability possible in the result, we have in certain instances made observations at four different temperatures. These new data are recorded below along with a few observations on the effect of sulfathiazole on the extra oxygen consumption associated with growth.

MATERIALS AND METHODS

The strain of *Serratia marcescens* used, the culture medium, and the methods of measuring the rate of oxygen consumption and of making the various calculations were all identical with those described previously (McLean and Fisher, 1947). The amount of nitrogen remaining in the medium after it had been separated from the bacteria by filtration was determined as before by digestion, aeration, and nesslerization.

It was desired in the present experiments to examine the metabolism of the bacteria with carbon and nitrogen sources other than those on which the cultures were grown routinely. It was therefore necessary to make sure that the small quantities of nutrients washed off the culture slants along with the organisms were removed before the experiment proper began. To accomplish this, the suspension of organisms obtained by washing off the plates into 0.07 M potassium

phosphate buffer at pH 7.0 was aerated at 30 C for approximately 3 hours before the respiratory experiment was begun. A small quantity of citrate was added at the beginning of the aeration. In the presence of this excess of citrate the organisms removed all of the nitrogen source (ammonia), and in due course the citrate itself disappeared. At this point the rate of oxygen consumption fell to the low value that is characteristic of cells deprived of both carbon and nitrogen sources. This rate is generally called the endogenous rate.

Each respirometer vessel contained: (1) 1-ml aliquot of a bacterial suspension the respiration of which had been reduced to the endogenous level as just described (it contained approximately 10^9 bacteria); (2) 0.5 ml of 0.4 per cent magnesium sulfate; (3) 0.25 ml of the carbon source as a 4 to 8 per cent solution; and (4) 0.25 ml of the nitrogen source which contained 0.025 to 0.05 mg N_2.

Whenever necessary the pH of the solutions was adjusted to 7.2 by adding hydrochloric acid or sodium hydroxide. After the equilibration period the rate of oxygen consumption was followed, and when it had become constant (after 30 to 90 minutes) at the "resting rate," the nitrogen compound was added from the onset and the oxygen uptake was observed for several hours more.

RESULTS

Preliminary experiments. The medium in which the organisms were suspended at the start of these experiments, containing, as it did, phosphate buffer, magnesium sulfate, and a carbon source, lacked only a source of nitrogen in order to support the growth of the organisms. If the nitrogen-containing compound which was added could be utilized by the organisms, then growth ensued upon the addition of this substance and the mass of bacterial protoplasm in the vessel began to increase. This in general causes the rate at which oxygen is taken up in the respirometer to increase, and, as we and others have shown, under suitable circumstances the rate of growth can actually be determined from the rate of the oxygen uptake in the vessel.

The addition of a number of possible nitrogen sources to the resting bacteria in the respirometer did not cause a rise in the rate at which oxygen disappeared. In these instances, therefore, it was considered that growth had not occurred and, consequently, that the respective combinations of carbon and nitrogen sources employed would not support rapid growth. It was ascertained in this way that, with the mixture of citrate and glycerol used in our previous work as the carbon source, no growth took place in a 3-hour period after the addition of any one of the following as the sole source of nitrogen: creatine, glycine, leucine, D-glutamic acid, L-histidine, L-cystine, methionine, phenylalanine, tyrosine, or tryptophan. On the other hand, with ammonium chloride as the nitrogen source, growth was not supported by the carbon sources tartrate, acetate, succinate, alcohol, or lactate.

Rapid growth was found with various combinations of the nitrogen sources—asparagine, urea, and DL-alanine—and the carbon sources—citrate, glycerol, and pyruvate, or the mixture of citrate and glycerol.

With aspartic acid as the nitrogen source only very slow growth took place.

However, asparagine, the amide of aspartic acid, supported rapid growth. Analyses of the medium in the asparagine experiment showed, too, that all of the nitrogen of asparagine (i.e., both the amine and the amide nitrogen) was used. It was therefore of interest to find that, even in the presence of added ammonia, the amine nitrogen was still taken up very slowly.

Nitrogen and carbon sources. We pass now to a detailed consideration of the observations made using compounds that supported growth. These data are best represented by curves that show the cubic millimeters of oxygen taken up
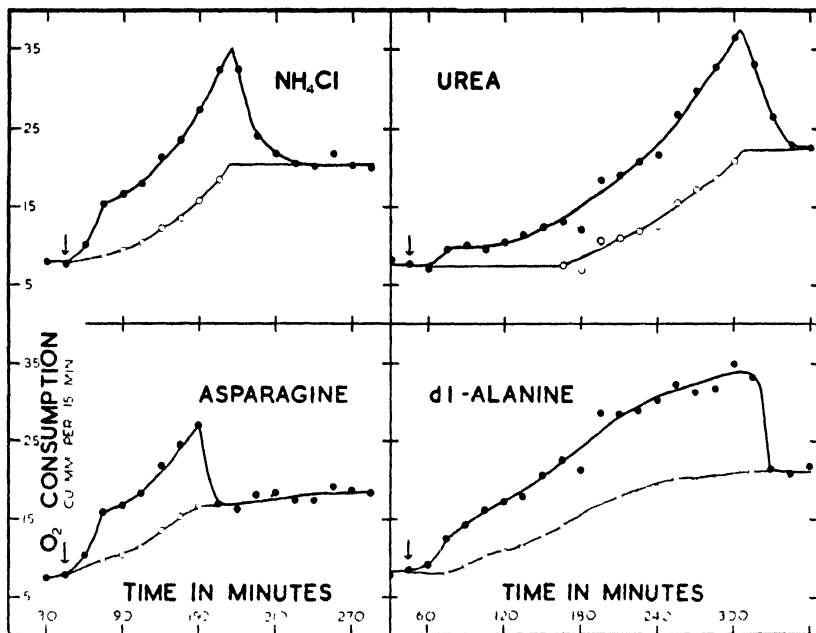
Figure 1. The time course of the rate at which oxygen is taken up in a respirometer vessel containing *S. marcescens* suspended in the nonnutrient medium to which 0.05 mg of nitrogen were added as ammonium chloride, urea, asparagine, or DL-alanine at the time marked by the arrow. Each experimentally determined point (indicated by a dot) is the average of the values from 8 to 10 separate experiments. The open circles indicate the calculated resting rates corresponding to the rates observed on the growing cells. This calculation was made exactly as described by McLean and Fisher (1947).

per vessel per 15 minutes plotted against time. Typical data using ammonium chloride (these from our previous work), urea, asparagine, or DL-alanine as the nitrogen source, with the mixture of citrate and glycerol as the carbon source, are shown as dots in figure 1. The results for the carbon sources, citrate, glycerol, and pyruvate, with ammonium chloride or asparagine as the nitrogen source are not shown as they are qualitatively identical with the ones using citrate plus glycerol and differ only quantitatively from them.

The course of the oxygen uptake following the addition of the nitrogen source is strikingly similar in all these cases. It may be seen (figure 1) that during the

20 to 30 minutes following the addition of the nitrogen source the rate of oxygen uptake increased by 50 to 100 per cent. Thereafter the rate of oxygen continued to rise, but it did so somewhat less rapidly. The initial rapid rise followed by the more gradual one was so constant and striking a feature of these experiments that to emphasize it the lines have been drawn through the points in figure 1 as if the transition from one phase to the other occurred instantaneously at a definite moment in time (which, of course, was undoubtedly not strictly the case). The rate of oxygen uptake continued to rise during the second phase and finally it reached a maximum from which it fell sharply to a reasonably constant lower value. The sharpness of the fall was so striking (it frequently occurred between successive readings of the respirometer) that again the lines have been drawn through the points as if the transition from the rising rate of oxygen consumption to the falling one occurred instantaneously, which undoubtedly was not quite the case.

In our previous experiments with ammonium chloride it was shown that, as nearly as one could see, the disappearance of the last traces of the ammonia from the medium coincided with the occurrence of the maximum rate of oxygen uptake. Chemical analyses of the medium in the present experiments indicated precisely the same thing: exhaustion of the nitrogen source was associated with a fall in the rate at which oxygen is taken up in the vessel. Now growth, as indicated by the uptake of the nitrogen source, must have ceased at the moment that all of the nitrogen was taken up, so that from this moment the quantity of bacterial protoplasm present in the vessel must have been constant. We have already noted that the rate of oxygen uptake fell to a lower constant value following the cessation of growth. This must mean that the bacterial protoplasm consumed oxygen at two rates, depending upon whether it was actively growing or was in a resting condition.

The relative magnitudes of the two rates, the resting and the growing, are indicated by the maximum rate of oxygen uptake found in the respirometer and the lower relatively constant rate found some time after this. It is convenient to express the resting rate as a percentage of the growing one. Its value is given by $\dfrac{\text{constant rate which follows maximum rate}}{\text{maximum rate}} \times 100$. It was shown in our earlier investigation (McLean and Fisher, 1947), in which ammonium chloride was used as the nitrogen source and a mixture of citrate and glycerol as the carbon source, that the resting rate averaged 57 per cent of the growing one. This value was obtained even when the amounts of growth in the respirometers were varied by varying the amount of ammonia added to the initially resting cells. This observation was of particular significance because it indicated that for each growing rate of oxygen consumption there was a corresponding resting rate to which the oxygen consumption would fall if the ammonia present were suddenly withdrawn. Therefore it is possible to include in addition to the measured rates (the dots) in figure 1 a set of calculated rates (the circles), which show the resting rate to which the oxygen consumption would have fallen if at any moment the ammonia in the medium had been suddenly removed. It will be

noted that the line traced out by these calculated points indicates the way the rate of oxygen uptake in the respirometer would have changed with time if the amount of bacterial protoplasm in the respirometer had been gradually increased without the participation of the normal chemical processes of growth, as, for example, by the gradual addition of more and more resting cells.

The area that is bounded by the line showing the course of the observed growing rate and the calculated line describing the resting rate represents a quantity of oxygen. It is the amount of oxygen that the bacterial protoplasm consumed during growth in excess of that which would have been used by the same protoplasm at rest. More specifically still, it is the amount of oxygen associated with a definite amount of growth, namely, that growth which involved the assimilation of a known quantity of a known nitrogen-containing compound. As was pointed out in our earlier paper, it is of interest to consider this quantity of oxygen in relation to the quantity of nitrogen assimilated, for it represents the *cost* of growth under these conditions in terms of oxygen.

These considerations relating to the growing and resting rates were developed initially with respect to our earlier experiments in which only ammonia was used. It appears, however, that they must apply equally well to these new experiments in which nitrogen was supplied as urea, asparagine, or alanine. The time course of the resting rates shown in figure 1 for the urea, asparagine, and alanine experiments was in fact calculated in exactly the same way as in the original work using ammonium chloride.

Although these observations are qualitatively the same as the previous ones, they differ quantitatively from them. A glance at figure 1 reveals the areas to be different. Actually, for quantitative comparisons of the results observed under the various circumstances described above, the data permit four measurements to be made (McLean and Fisher, 1947): (1) the generation time, (2) the resting rate as a percentage of the maximum, (3) the difference between the resting rate before the nitrogen source was added and the resting rate after the nitrogen source was exhausted, and (4) the area that measures the extra oxygen associated with assimilation of the nitrogen source. These will be discussed in turn.

The generation time is the time for the bacterial mass to double. It was taken as the time required during the logarithmic phase for the rate of oxygen consumption to double. This procedure is justified by our previous observation (McLean and Fisher, 1947) that, under these conditions at least, the rate of increase in either the growing or the resting rate of oxygen consumption is strictly proportional to the rate of increase of the bacterial mass. The average values of the generation time found using various nitrogen and carbon sources are shown in table 1, column 3. They are clearly not identical: with ammonium chloride, citrate, and glycerol growth was very rapid, i.e., the generation time was short, whereas with pyruvate or asparagine and the same carbon sources the growth was considerably slower. Marked differences in the generation times do not appear here since compounds in which growth was very appreciably slower were intentionally excluded. Special mention must be made of DL-alanine

TABLE 1

Quantitative characteristics of the growth of S. marcescens as functions of the nature of the carbon and nitrogen sources

N SOURCE (1)	C SOURCE (2)	GENERATION TIME Minutes (3)	RESTING RATE OF O_2 CONSUMPTION As % of growing rate (4)	INCREASE IN RESTING RATE ml O_2/hr/mg N (5)	O_2 ATOMS Per atom N (6)
Ammonium chloride	Citrate + glycerol	72 ± 6 (10)	57.0 ±1.8 (1)	1.02 ±.06 (10)	2.17 ± .14 (10)
	Citrate	98 ± 8 (10)	60.0 ±3.6 (7)	1.31 ±.34 (6)	3.05 ± .35 (7)
	Glycerol	88 ±13 (7)	56.5 ±5.4 (6)	1.43 ±.31 (6)	3.65 ± .47 (6)
	Pyruvate	100 ± 7 (10)	71.6 ±3.6 (10)	1.14 ±.26 (10)	1.90 ± .16 (10)
Asparagine	Citrate + glycerol	78 ± 8 (10)	68.3 ±4.3 (10)	0.92 ±.14 (10)	1.06 ± .14 (10)
	Citrate	95 ±17 (10)	62.0 ±2.8 (10)	1.20 ±.14 (10)	2.14 ± .19 (10)
	Glycerol	88 ±15 (10)	69.1 ±2.6 (10)	1.00 ±.20 (10)	1.57 ± .26 (10)
	Pyruvate	116 ±11 (9)	70.0 ±4.2 (9)	0.94 ±.17 (9)	1.80 ± .18 (9)
Urea	Citrate + glycerol	99 ±15 (10)	57.6 ±3.6 (9)	1.23 ±.10 (10)	3.38 ± .11 (8)
Alanine	Citrate + glycerol	approx. 66 (10)	61.9 ±3.7 (9)	1.03 ±.07 (10)	3.80 ± .12 (8)

The standard deviation of each quantity is given following the ± sign. The number of experiments made to obtain each quantity is given in parentheses following it. The value for the generation time with alanine as the nitrogen source is estimated, since, as indicated in the text, a logarithmic phase of growth was not observed with this compound.

(figure 1), for with this mixture of isomers no phase was observed during which the logarithm of the rate of oxygen consumption was a linear function of time. Nevertheless the rate of oxygen consumption did increase rapidly when alanine was added, and after increasing to a maximum it fell to a relatively constant resting rate. As chemical analyses showed that both of the enantiomorphs were utilized, the departure from logarithmic growth was probably due to a difference in the rates at which the two were consumed.

The final resting rate of oxygen consumption (i.e., that relatively constant value observed when nitrogen assimilation is finished), when expressed as a percentage of the maximum rate of oxygen consumption in a given respirometer, provides an indication of the relative magnitudes of the growing and resting rates of oxygen consumption (Armstrong and Fisher, 1947; McLean and Fisher, 1947). The values found for the different nitrogen and carbon sources are given in column 4 of table 1. They vary from 57 to 71 per cent, indicating that the value of the resting rate was a function of the carbon and nitrogen sources being utilized. Speculation concerning the reason for this does not seem warranted at present, however, since the actual values may not be as accurately determined as the standard deviations would tend to indicate. This follows from the fact that the curves relating the rate of oxygen consumption to time vary slightly from one compound to another. For example, after the assimilation of asparagine (figure 1), the resting rate rose gradually for a time, whereas after the assimilation of ammonia it fell gradually. Such differences in the shapes of these curves may conceivably have led to small systematic errors in the calculations made from them.

Since the resting rate of oxygen consumption has been found to be proportional to the amount of bacterial nitrogen present (McLean and Fisher, 1947) the increase in this rate after the assimilation of a certain quantity of a nitrogen source provides an estimate of the increase in the bacterial protoplasm. The values for this increase after the assimilation of 1 mg of nitrogen are given in column 5 of table 1. Although they are not all identical (those for asparagine, for example, were generally slightly lower than the others), one is much more impressed with the similarity of the values than with the differences between them. This is especially true when it is recalled again that there were small differences in the shapes of the curves from which the resting rates were determined. At least to a first approximation then, one may conclude that the increase in level did not depend on the source of either the carbon or the nitrogen. The presumption is therefore that all of the nitrogen in each case was incorporated into protoplasm and not stored in any relatively inert form.

The atoms of oxygen consumed per nitrogen atom assimilated were calculated in each instance, as described earlier, from the area bounded by the lines representing the change of the growing and resting rates (see figure 1). This area is, of course, a function of the generation time, of the difference between the growing and resting rates of oxygen consumption, and of the increase in the resting rate due to the assimilation of a given quantity of nitrogen. Other things being equal, an increase in the generation time will result in an increase in the area.

If the difference between the growing and resting rates increases, this too will increase the area. Also, if the increase in the resting rate is greater with one compound than with another, the area will increase because a longer time will be required to consume the necessary oxygen.

The individual factors upon which the experimentally determined areas depend have already been found to vary from one compound to another. It follows that unless the variations of the factors occurred in a reciprocal fashion the areas will also vary. The latter have been determined in the present research. They have been expressed in terms of the number of oxygen atoms consumed for each atom of nitrogen assimilated and will be found in table 1, column 6. Large differences between the values are apparent immediately, so that the factors that determine the areas certainly did not vary with one another in a reciprocal fashion. It is evident that the oxygen consumed during the assimilation of a particular nitrogen source varied with the carbon source available, and conversely that with a given carbon source, the mixture of citrate and glycerol for example, the amount of oxygen consumed varied with the nitrogen source. It is therefore clear that both the nitrogen and the carbon source had a marked influence on the quantity of oxygen involved in the growth process.

Although the values recorded in the table appear to vary somewhat randomly, two regularities are suggested. First, the number of oxygen atoms consumed per nitrogen atom assimilated as asparagine was always equal to or less than that required if the nitrogen was supplied as ammonia. Second, the oxygen consumed per nitrogen atom assimilated was greater when either citrate or glycerol alone was the carbon source than it was when both of these compounds were present simultaneously. Evidently some sort of interaction was possible when both carbon sources were present.

Temperature. To test the effect of temperature on the growth that resulted when the nitrogen source was added, experiments exactly like those reported in figure 1 were conducted simultaneously on aliquots of the same bacterial suspension in baths at 26, 34, and 38 C, respectively. As before, citrate, glycerol, $MgSO_4$, and the bacteria suspended in buffer were in the main space of the vessel, and ammonium chloride or asparagine was added from the onset. Since the observations discussed earlier were made at 30 C, there are available, in all, measurements at four temperatures, 26, 30, 34, and 38 C. As would be expected, the generation time varied with the temperature (table 2, column 3), and the data suggest that the growth rate reached a maximum under these conditions at approximately 34 C.

The fact that the resting rate of oxygen consumption would become a larger fraction of the growing rate as the temperature rose (table 2, column 5) probably could not have been predicted a priori. It will be noted that this effect of temperature was much more marked when the nitrogen source was ammonia than when it was asparagine.

The values for the increase in the resting rate of oxygen consumption due to the assimilation of 1 mg of nitrogen are given in column 6 of table 2. As would be expected, the amount by which the resting rate increased at a given tempera-

TABLE 2

Quantitative characteristics of growth of S. marcescens as functions of temperature with each of two sources of nitrogen

N SOURCE (1)	TEMP (C) (2)	GENERATION TIME Minutes (3)	Q_{10} (4)	RESTING RATE OF O_2 CONSUMPTION As % of growing rate (5)	INCREASE IN RESTING RATE ml O_2/hr/mg N (6)	Q_{10} (7)	O_2 ATOMS PER ATOM N (8)
Ammonium chloride	26	98 ±17 (9)	3.3	54.4 ±3.6 (9)	6.4 ±0 8 (9)	3.1	2.32 ±0.25 (9)
Ammonium chloride	30	72 ± 6. (10)		57 0 ±1.8 (10)	10.1 ±0.43 (10)		2 17 ±0.14 (10)
Ammonium chloride	34	71 ± 3.5 (9)	0.15	62.8 ±2.6 (9)	11 3 ±1.43 (9)	1.3	1.95 ±0.22 (9)
Ammonium chloride	38	90 ±14.5 (9)		73.1 ±3 8 (9)	12.3 ±1.25 (9)	1.0	1.61 ±0.30 (9)
Asparagine	26	102 ±23. (8)	2.9	66.4 ±2 9 (9)	6.2 ±0.66 (9)	4.2	1.13 ±0 17 (9)
Asparagine	30	78 ± 7.5 (10)		68.3 ±4 3 (10)	9.1 ±1.28 (10)		1.06 ±0.14 (10)
Asparagine	34	74 ±12. (10)	0.6	69.5 ±3.0 (9)	10 5 ±0.91 (9)	1.6	1 28 ±0.25 (9)
Asparagine	38	78 ± 5. (9)		73.0 ±3 6 (8)	10.1 ±1.94 (8)		1.18 ±0.21 (8)

As in table 1, the figures in parentheses indicate the number of separate determinations averaged to obtain the value shown, and the standard deviation of the separate determinations about the average is given following the ± sign.

ture was approximately the same whichever nitrogen source was involved. It therefore follows that the data given in the table represent actually the resting rate of oxygen consumption per mg of bacterial nitrogen as a function of temperature. The temperature coefficients in table 2, column 7, indicate therefore the effect of temperature on the resting rate of oxygen consumption.

The values for the extra oxygen consumed for each nitrogen atom assimilated, which will be found in table 2, column 8, were essentially independent of temperature when asparagine was the nitrogen source, but varied somewhat when

TABLE 3

Quantitative characteristics of the growth of S. marcescens in the presence and absence of sulfathiazole (ST)

(The ST concentration was 0.05 per cent)

	AMMONIUM CHLORIDE			ASPARAGINE		
QUANTITY DETERMINED	No ST	ST		No ST	ST	
		Absolute	% Control		Absolute	% Control
(1)	(2)	(3)	(4)	(5)	(6)	(7)
Increase in resting rate due to growth (ml O_2/hr/mg N).......	1.02 (0.05)	0.92 (0.08)	90	0.92 (0.14)	0.78 (0.10)	84
Generation time (minutes).......	72 (6)	124 (14)	168	78.0 (7.5)	114 (17)	
Relative growth rate (from generation time)...	100	58	58	100	68	68
Level as % peak..	56.4 (0.9)	71.0 (1.2)	—	68.0 (4.2)	78.5 (6.0)	—
$\frac{\text{Growing-resting}}{\text{resting}} \times 100$..........	77.3	40.8	53	47	27.5	59
Oxygen atoms per nitrogen atom..	2.19.(0.14)	1.61 (0.11)	73.5	1.17 (0.31)	0.83 (0.17)	71

The quantities of the nitrogen sources added were sufficient to provide initially 0.05 mg of nitrogen per respirometer vessel. Each control (i.e., no ST) value is the average of 10 determinations, and each value with ST present is the average of 6. The standard deviation of the separate determinations from each average is given in parentheses following it.

the nitrogen source was ammonium chloride. Although the changes with temperature in the latter case were small, their regularity leaves little doubt about their reality. It seems necessary to conclude that the chemical mechanism by which ammonia was assimilated varied slightly with the temperature.

Sulfathiazole. The increased rate of oxygen consumption with the assimilation of nitrogen, i.e., with growth, in *E. coli* has already been found to be inhibited by sulfathiazole (Armstrong and Fisher, 1947). Since a similar increase in the rate of oxygen consumption takes place when *S. marcescens* is growing, the effect of sulfathiazole on the oxygen consumption of this organism was also

investigated. The present experiments were primarily designed to show the effect of sulfathiazole on the number of oxygen atoms needed in the ammonia assimilation, as well as the effect on the rate of oxygen consumption during assimilation, which was already known for *E. coli*. In these experiments the bacteria were prepared with citrate plus glycerol as the carbon sources. Sulfathiazole was added with these when desired to give a final concentration of 0.05 per cent. After the resting level had been established, a limited amount of ammonium chloride or asparagine was added and the rate of oxygen consumption was followed as usual. The quantitative aspects of the curves showing the rates of oxygen consumption with and without sulfathiazole are compared in table 3. The data for ammonium chloride and asparagine are essentially identical. They show that, while there was only a slight inhibition (10 per cent) of the resting rate, the generation time was markedly increased (i.e., the rate of growth was decreased) by sulfathiazole and that the rate of oxygen consumption during growth was much lower in the presence of sulfathiazole. These results confirm those of Armstrong and Fisher for *E. coli* under similar conditions.

It will be noted in table 3 that the amount of oxygen consumed for each nitrogen atom assimilated was less in the presence of sulfathiazole than in its absence. One must conclude from this new observation that sulfathiazole caused some qualitative change in the sequence of chemical reactions by which ammonia and asparagine were taken up.

DISCUSSION

It is of interest to consider the quantity of oxygen consumed during the assimilation of the various nitrogen sources in terms of some of the chemical changes that might be involved. Suppose, for example, that the first step in the assimilation of the nitrogen in alanine is an oxidative deamination. This would, of course, require 1 atom of oxygen for each molecule of alanine destroyed. The utilization of each molecule of ammonia thus set free would require, according to column 6 of table 1, 2.2. atoms of oxygen, if carbon were supplied as the mixture of citrate and glycerol. In all, therefore, 3.2 atoms of oxygen would be taken up for each atom of nitrogen utilized. From column 6 of table 1 it may be seen that 3.8 atoms of oxygen were actually required. The difference between 3.2 and 3.8 seems appreciable, although it may not be very significant.

In the case of urea it appears possible that ammonia is first formed by the hydrolytic action of urease, and that the only oxygen needed is that used during the assimilation of this ammonia. If so, no more oxygen would be needed for the assimilation of urea than for the assimilation of ammonia itself. However, the data indicate that, in the citrate and glycerol mixture, 2.2 atoms of oxygen are required to assimilate 1 nitrogen atom in the form of ammonia, whereas 3.4 are required if the nitrogen is supplied as urea. One therefore infers that an oxidative breakdown of the urea must take place, rather than hydrolysis.

The assimilation of asparagine is of particular interest since less oxygen was taken up here than in any other of the cases examined (table 1, column 6). If

the amine nitrogen of this molecule were utilized in the manner already considered for alanine, then 3.2 atoms of oxygen would be taken up for each amine group which disappeared. As a consequence, even if no oxygen were required for the assimilation of the amide nitrogen, 1.6 atoms of oxygen would disappear for each nitrogen atom taken up. Actually, in the citrate and glycerol mixture, only 1.1 atoms of oxygen were required, so that oxidative deamination presumably is not the actual mechanism.

It is of course possible that the utilization of asparagine involved first a hydrolysis of the molecule to ammonia and aspartic acid, a reaction known to occur in a variety of organisms. Provided that the aspartic acid, left perhaps in a particularly reactive state, did not require any oxygen, and that the utilization of the ammonia required the usual 2.2 atoms per nitrogen atom, this would result in the consumption of 2.2 atoms of oxygen per asparagine molecule, or 1.1 atoms per nitrogen atom. This would account for the observations with asparagine in the citrate and glycerol mixture but clearly not when other carbon sources were used.

These considerations thus make it clear that it is not practical at present to propose a complete chemical scheme that will summarize the new observations recorded in this paper.

SUMMARY

The oxygen consumption of *Serratia marcescens* was studied during growth and also with the cells in a resting condition.

The rate of oxygen consumption by growing cells of *Serratia marcescens* is significantly higher than that of resting cells when growth is supported by such nitrogen sources as ammonia, alanine, urea, or asparagine with carbon supplied as citrate, glycerol, or pyruvate.

The quantity of oxygen associated with the assimilation of known quantities of each nitrogen source was determined. It was found to vary over a threefold range depending upon the temperature and the nature of both the nitrogen and carbon sources. It was likewise modified by adding sulfathiazole.

The observations are discussed briefly in relation to some of the current ideas about the chemical changes which might be involved.

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ACID FUCHSIN METHYLENE BLUE AGAR: A NEW DIFFERENTIAL MEDIUM FOR ENTERIC BACTERIA

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Despite the development of successful *selective* media favoring the more pathogenic members of the *Enterobacteriaceae*, media of primarily *differential* nature have not been entirely satisfactory for several reasons, such as the following: (1) Generally only colonies of lactose fermenters are colored, whereas the colorless colonies of nonfermenters tend to be masked by the diffusion of dye from fermenting colonies (Holt-Harris and Teague, 1916). Though two dyes are employed in eosin methylene blue agar, a chemical combination occurs to yield the dye methylene blue eosinate (Wynne *et al.*, 1942), which is absorbed by fermenters only. Since methyl green resembles methylene blue in having a quinoid structure with a pentavalent nitrogen atom, a similar combination is chemically feasible in the recently devised eosin methyl green sulfite agar of Fredericq (1947). Furthermore, the color differentiation obtained with this medium is compatible with such a reaction between the dyes. (2) The medium may be too toxic for the growth of delicate enteric species (Fredericq, 1947). (3) Deterioration may occur on exposure to light (Endo, 1904). (4) Weak fermenters may not be differentiated from nonfermenters (Lederburg, 1948). The recent use of tetrazolium indicator by Lederburg (1948) seems to have largely obviated the first three difficulties, but the problem of weak fermenters is still troublesome.

There are, nevertheless, many types of work in which *differential* media are of vital importance, particularly certain studies on antagonistic coliform strains (Wynne, 1947; Halbert, 1948*a,b*). Acid fuchsin methylene blue (AFMB) agar was formulated in an attempt to provide a more satisfactory differential medium essentially free from the objections listed above. A mixture of an acid and a basic dye was used on the well-known theory of Stearn and Stearn (1924) that colonies producing acid would tend to be colored by the former and those not producing acid by the latter.

METHODS

The basal medium used for testing the various dyes used in this investigation contained, per liter: Difco peptone, 10 g; lactose, 10 g; K₂HPO₄, 2 g; agar, 15 g. All dyes used were certified by the Biological Stain Commission (Conn, 1946).

The organisms used, with the exception of the enterococci, were secured from the stock culture collection of the Department of Plant Sciences at the University of Oklahoma. For preliminary testing of dyes, *Aerobacter aerogenes* and *Escherichia coli* were employed as lactose fermenters and *Shigella dysenteriae*

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Shiga and *Alcaligenes faecalis* as nonfermenters. The last two organisms are known to be sensitive to basic aniline dyes (Kligler, 1918). The biochemical characteristics of the four organisms corresponded to those given by *Bergey's Manual* (1948). Incubations were at 37 C.

<div align="center">RESULTS</div>

The acid dyes tested were acid fuchsin, Congo red, and picric acid. The basic dyes included Bismark brown Y, crystal violet, malachite green, methyl green, methylene blue, nigrosin, thionin, and toluidine blue O. Acid fuchsin and methylene blue were selected as being most satisfactory from the standpoint of low toxicity and differential value.

However, the production of red colonies by lactose fermenters and blue colonies by nonfermenters in a medium containing a *mixture* of these two dyes would be possible only if the dyes do not combine chemically (Wynne *et al.*, 1942). The filter paper adsorption technique of Clifton and Madison (1931) was applied to 1 per cent aqueous solutions of acid fuchsin and methylene blue and to a mixture containing 1 per cent of each dye. With a solvent rise of 4 cm, acid fuchsin alone showed practically the same elevation as the solvent, whereas methylene blue rose only about 1 cm. In the mixture, each dye clearly behaved as if it alone were present. Additional evidence of a lack of combination was obtained by filtration (Wynne *et al.*, 1942) through Mandler candles without negative pressure. Acid fuchsin appeared in the first 2 to 4 ml of filtrate, but methylene blue appeared only after approximately 80 ml. A mixture of the two again showed acid fuchsin in the first 2 to 4 ml, but methylene blue could be detected only after approximately 140 ml were filtered.

Optimum concentrations of acid fuchsin and methylene blue in the medium were found to be 1:2,000 and 1:20,000, respectively. The pH recommended is 6.6, since at values appreciably higher dye-sensitive species such as *S. dysenteriae* Shiga and *A. faecalis* fail to develop. Such behavior is in accordance with the known increase in adsorption, and therefore in toxicity, of methylene blue with increasing pH (Stearn and Stearn, 1924). AFMB agar is lavender violet (Ridgway, 1912) in color at pH 6.6. Since acid fuchsin is decolorized at a pH around 7, at which the medium is a clear blue, direct colorimetric adjustment of the pH may be done with a little practice.

Optimum buffer concentration was shown to be 0.3 per cent. Lower concentrations tend to allow masking of nonfermenting colonies by those producing acid, whereas higher concentrations may prevent ready differentiation of weak fermenters.

In agreement with adsorption and filtration studies, lactose fermenters produce red colonies on AFMB agar, and nonfermenters produce blue colonies. Generally, transmitted light is preferable for viewing purposes, though reflected light is at times quite helpful. When a mixture of the two colony types is viewed by transmitted light and the plate moved slowly out of the direct path of light, colonies of nonfermenters seem to fade somewhat, but fermenting colonies remain prominently red. Incubation should be 18 to 24 hours for optimum differentiation.

AFMB agar was compared with Levine's (1918, 1943) EMB agar (pH 7.2) with 20 strains of enteric bacteria (table 1). In view of the critical nature of the

TABLE 1

Comparative toxicity studies with AFMB and EMB agar

ORGANISM	EMB (pH 7.2)	AFMB (pH 6.6)
Aerobacter aerogenes .	+	+
Alcaligenes faecalis	+	+
Escherichia coli..	+	+
Escherichia freundii.	+	+
Paracolobactrum aerogenoides	+	+
Paracolobactrum coliforme	+	+
Proteus ammoniae 29	+	+
Proteus ammoniae 30	+	+
Proteus OX19	−	+
Proteus vulgaris.	−	+
Salmonella enteritidus	+	+
Salmonella paratyphi	+	+
Salmonella pullorum	+	+
Salmonella schottmuelleri	+	+
Salmonella typhimurium	+	+
Salmonella typhosa	+	+
Shigella dysenteriae Shiga	−	+
Shigella paradysenteriae 1	+	+
Shigella paradysenteriae 2	+	+
Shigella paradysenteriae (Newcastle)	−	+

+ = growth. − = no growth.

TABLE 2

Recovery of lactose fermenters and nonfermenters from mixtures

ORGANISMS	NO. COLONIES OF FERMENTERS/NO COLONIES OF NONFERMENTERS	
	EMB	AFMB
E. coli and *S. typhosa*	200/100	200/100
A. aerogenes and *S. typhosa*	180/30	190/70
E. coli and *S. dysenteriae* Shiga	150/0	220/40
A. aerogenes and *S. dysenteriae* Shiga	100/0	150/30
A. aerogenes and *S. sonnei*	120/110	100/100
E. coli and *Proteus* OX19	120/4	125/60
A. aerogenes and *Proteus* OX19	100/2	140/60
E. coli and *S. typhimurium*	90/250	120/300
A. aerogenes and *S. typhimurium*	100/300	100/250

pH in AFMB agar, the EMB medium was retested at a pH of 6.6 with the four inhibited species, but only *Proteus* OX19 grew.

The greater toxicity of EMB agar was also evident in streaking mixtures of lactose-fermenting and nonfermenting strains. The semiquantitative technique of Wynne (1947) was used, and the data in table 2 show a definite superiority

of the AFMB medium with mixtures containing *Proteus* OX19 and *S. dysenteriae* Shiga.

Weak fermenters were also more easily detected on AFMB agar. A strain of *Escherichia freundii* showing retarded lactose fermentation gave distinctly red colonies on the AFMB medium after 18 hours' incubation, but the colonies on EMB agar were colorless even after 24 hours. Eight strains of freshly isolated enterococci likewise produced red colonies on AFMB agar with colorless colonies on EMB at 24 hours' incubation. It is a matter of common experience that enterococci are often mistaken for lactose-negative *Enterobacteriaceae*.

SUMMARY

A new differential medium for enteric bacteria, AFMB agar, with the following composition is described: Difco peptone, 10 g; lactose, 10 g; K₂HPO₄, 3 g; acid fuchsin, 0.5 g; methylene blue, 0.05 g; agar, 15 g; and distilled H₂O to 1,000 ml; pH 6.6.

Lactose fermenters produce red colonies, whereas nonfermenters are blue. Masking of the latter type by the former is minimized.

AFMB agar is less toxic than Levine's EMB agar and allows a more ready differentiation of weak fermenters.

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ALTERED RESPONSES OF NEUROSPORA CRASSA TO INHIBITING CONCENTRATIONS OF INDOLE[1]

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The discovery in *Neurospora crassa* of gene mutations that enable the organism to grow at enhanced rates in the presence of otherwise inhibitory concentrations of organic compounds (Emerson and Cushing, 1946; Horowitz and Srb, 1948) has shown that this mold has qualities favoring its use in the study of the genetic basis of adaptive phenomena. With this in view, efforts have been made to find other adaptive mutations in this organism.

Attention has been directed toward adaptations involving the inhibitory effects of high concentrations of metabolites normally produced during cellular metabolism in the hope that information might be obtained on the role of adaptive gene mutation in the regulation of the amounts of these compounds produced by the organism. It was also believed that such an approach might disclose the occurrence of specifically induced mutations of the type postulated by Emerson (1945), for it would seem reasonable to expect such mutations, if they occur, to form part of the regulative machinery of cells. This would seem to be true especially when regulation might involve compounds that the genetic machinery was already producing and conditioned to handling.

Material and methods. In conducting these studies a strain of "wild type" *Neurospora crassa* (E 5256A) originally obtained from Professor Sterling Emerson was used unless otherwise noted. Tests for inhibition by specific substrates and for subsequent adaptation were made in 125-ml Erlenmeyer flasks containing 20 ml of minimal medium (cf. McElroy, Cushing, and Miller, 1948, for further reference to the classical *Neurospora* techniques as used in this laboratory) plus the compound being studied. These flasks were sterilized by autoclaving, after adjusting the initial pH to 5.5 when necessary, and were incubated at 29 C $\pm$ 1 C unless otherwise noted.

Inhibition tests. Table 1 presents a summary of the tests made with various metabolites and shows that wild type *Neurospora* is in general quite resistant to inhibition by vitamins, amino acids, and various components of nucleic acid. This is in contrast to the effects of several of these compounds upon certain mutant strains of the same organism (e.g., Ryan, 1946; Bonner, Tatum, and Beadle, 1943) and also upon various bacteria (Porter, 1946). This differential effect still awaits a satisfactory explanation. (See also the observations of Horowitz, 1944,

upon the inhibitory action of DL-isovaline for preparations of the D-amino acid oxidase in *Neurospora*.)

After this preliminary survey was completed, attempts were made to adapt *Neurospora* to the inhibitory effects of *p*-aminobenzoic acid, DL-tryptophan, and indole.

As noted elsewhere (Cushing and Reid, 1948), continued growth through several transfers failed to disclose any adaptation to the inhibitory effects of *p*-aminobenzoic acid. Negative results were also obtained in the case of tryptophan, 12 successive subcultures in four parallel series failing to show enhanced growth when compared with untreated controls. Inhibition by 10^{-2} moles per

TABLE 1

Metabolites tested for ability to inhibit growth of wild type Neurospora crassa

A. Compounds noninhibitory at 10^{-2} molar concentration

Amino acids	*Nucleic acid components*	*Vitamins*
DL-Methionine	Ribonucleic acid	Thiamine
DL-Isoleucine	Desoxyribonucleic acid	Nicotinic acid
L-Leucine	Guanine hydrochloride	Nicotinamide
DL-*m*-Valine	Thymine	Pyridoxine
L-Tyrosine	Xanthine	Inositol
L-Cystine	Uracil	Choline
DL-Serine		Folic acid
DL-Norleucine		

B. Inhibitory compounds

	Effective concentration in moles per liter
Para-aminobenzoic acid* (Emerson and Cushing, 1946)	10^{-2} partial; 10^{-3} none
Anthranilic acid	10^{-2} complete; 10^{-3} slight
Indole-3-acetic acid	10^{-2} complete; 10^{-3} none
DL-Tryptophan* (Bonner and Tatum, 1944)	10^{-2} partial; 10^{-3} none
L-Tryptophan	10^{-2} partial; 10^{-3} none
Indole* (Tatum and Bonner, 1944)	10^{-2} complete; 10^{-3} strong; 10^{-4} very slight

* Refers to other reports for the inhibition of growth of wild type *Neurospora* by these compounds.

liter of DL-tryptophan results in a reduction of growth rate to one-half normal and the production of a red-brown pigment by the mold. (It may be significant that a similar pigment is produced by 3 biochemical mutations in *Neurospora* that require nicotinic acid or nicotinamide for growth—Bonner and Beadle, 1946.)

Adaptation to indole. In the case of indole inhibition, enhanced growth of the mold was noted in one of a pair of serial transfers at the end of the eighth conidial transfer. Comparative studies of the strain obtained from this culture (designated IA = indole-adapted) showed that it retained its wild type morphology but grew consistently faster than wild type strains upon 10^{-3} moles per liter of indole at various temperatures (figure 1). It should be noted that this concen-

tration of indole is comparable to that at which sulfanilamide begins to exert an inhibitory effect upon *Neurospora* (cf. Emerson and Cushing, 1946). At this time it was found through the use of seitz-filtered indole media that autoclaving did not affect the phenomena under discussion.
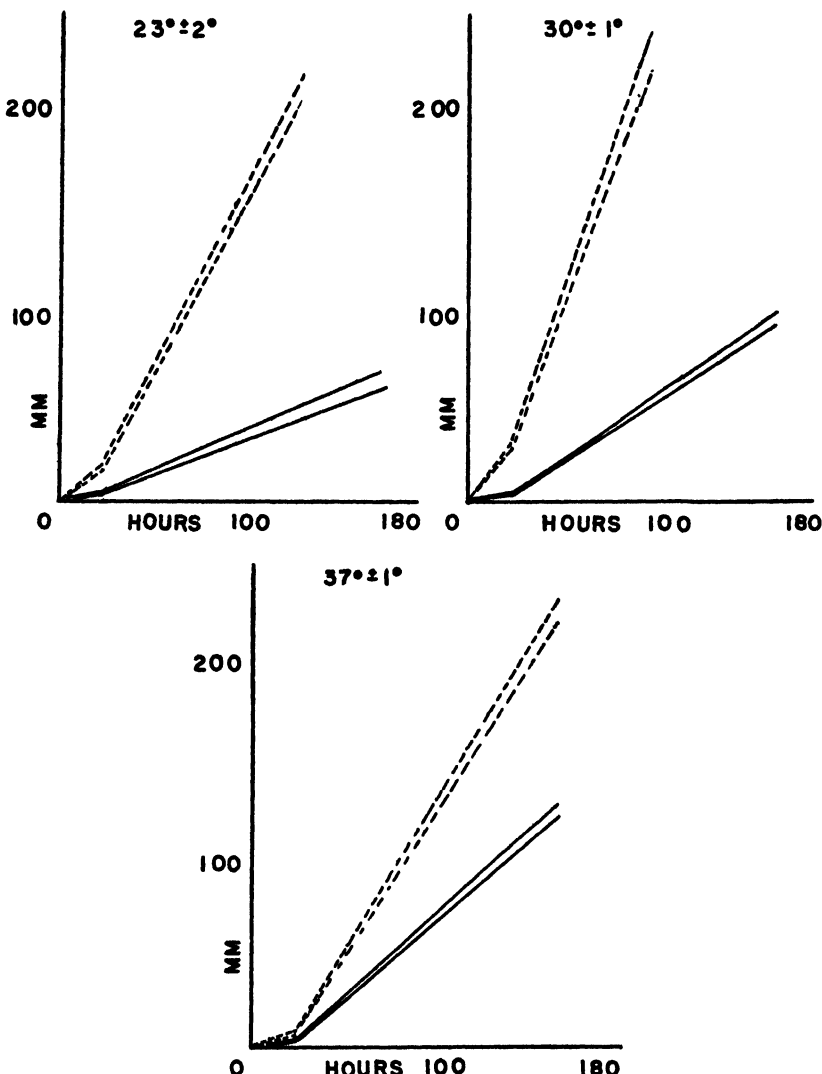
Figure 1. Growth of wild type (solid line) and indole-adapted (IA) strain (broken line) of *Neurospora* on solid minimal medium containing 10⁻³ moles of indole per liter.

The ability to grow at enhanced rates in the presence of indole persisted, not only through conidial transfer, but after growth through a long growth tube (200 mm) of minimal agar. This suggested that as in the case of the sulfanilamide-resistant strain a gene mutation was involved in the adaptation observed.

Genetics of indole adaptation. Verification of this point was obtained by crossing IA conidia with wild type protoperithecia. Two distinct types of strains were obtained from the resulting asci. These were (1) a wild type in appearance and physiology and (2) a mutant with characteristic morphological and physiological properties that will be further described.

Additional crosses were obtained between several of the mutant strains and wild type protoperithecia which show that the two types follow a segregation pattern typical for *Neurospora* of a pair of allelic genes located at a distance of roughly 25 crossover units from the centromere (11 out of 23 asci dissected in order showing segregations in the second meiotic division).

Description of the indole-resistant mutations. As noted above, the mutant allele of these crosses shows a distinctive morphology. Growth from the ascospore on the complete medium begins more slowly than does the wild type as a rough buttonlike growth that assumes essentially normal characteristics during the phase of active growth. As this phase ceases, yellowish sticky and compact aerial hyphae are formed that do not give rise to conidia. In addition, by this time droplets of a pinkish liquid have appeared at irregular intervals on the surface of the mycelium.

Associated with the foregoing morphology is the ability to grow at enhanced rates upon indole. As shown in figure 2 in which the growth rates of several wild type and mutant segregants are compared at 30 and 37 C, this ability is consistent at the lower temperature, but becomes more irregular at the higher. It is of interest to note that no isolates were obtained that grew as well on indole as the original IA strain. This suggests that IA exists in a balanced heterocaryotic state that preserves its wild type appearance and increases its powers of growth on indole after the fashion of the heterocaryons described by Emerson (1948) involving the sulfonamide-requiring strain of *Neurospora*. Attempts are being made to confirm this possibility, but to date reconstituted heterocaryons involving mutant strains and their wild type alleles have not been obtained.

A single strain (20-31) was selected as representative of the mutant type, and its growth rates on various concentrations of indole were compared with the IA and wild type strains at 30 C (table 2). The specificity of adaptation was also studied by growing 20-31 on several inhibiting compounds, and adaptation was found to be restricted to indole. The compounds and molar concentrations tested are as follows: sulfanilamide 10^{-3}, L-tryptophan 10^{-2}, anthranilic acid 10^{-2}, and indole-3-acetic acid 10^{-2} M. In these cases growth responses are the same as those reported for the wild type.

Mechanism of resistance. Tatum and Bonner (1944) have shown that *Neurospora* is capable of combining indole and serine to form tryptophan when both these compounds are present in the medium, and that this mechanism reduces the toxic effects of indole was noted by them. With this in mind, colorimetric measurement was made (Snell and Snell, 1937) of the amount of indole remaining in media after growth upon them of the IA and wild type strains of *Neurospora*. No changes in indole concentration were detected by this method, and it seems improbable that in this case adaptation is based upon an uptake of the
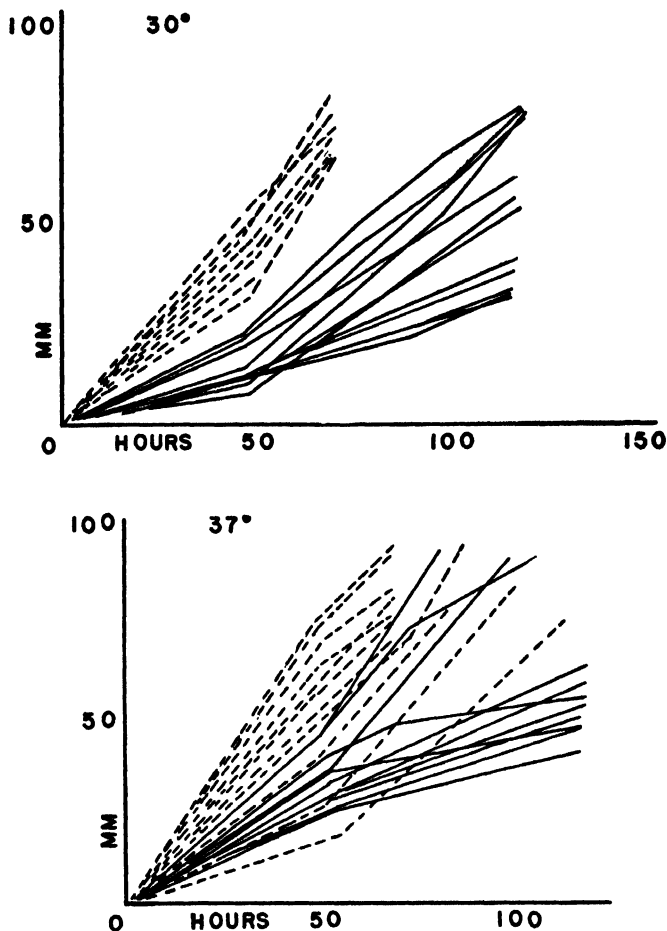
Figure 2. Growth of wild type and mutant segregants at 30 and 37 C on solid minima medium containing 10^{-3} moles of indole per liter. Dotted lines show range of variation among 24 mutant strains; solid lines show range of variation among 13 wild type strains. These strains were obtained as segregants from crosses between the original indole-adapted (IA) strain of *Neurospora* and the wild type.

TABLE 2

Effects of concentration of indole on the growth rate of wild type Neurospora, 20-31, and 1A, at 31 C

CONCENTRATION OF INDOLE IN MOLES PER LITER	AVERAGE GROWTH RATE (MM PER HOUR)		
	E 5256A	20-31	IA
$10^{-2.4}$	0.00	0.00	0.00
$10^{-2.7}$	0.11	0.80	0.78
$10^{-3.0}$	0.65	2.18	2.51
$10^{-3.3}$	2.20	3.00	3.6
Minimal Fries medium	4.5	3.00	3.5

indole molecule to form tryptophan as observed by Tatum and Bonner. However, in connection with the search for the mechanism involved in the adaptation it was observed that wild type *Neurospora* is much less inhibited by indole when growing on complete medium. The component of the complete medium involved is still unknown, but it occurs in both malt and yeast extracts, appearing to be more abundant in the former material (figure 3). At the same time that these observations were made, the mutant strain 20-31 was found to be inhibited by unknown materials in the yeast and malt fractions of complete media. This inhibition, though definite, was not so marked as that of indole upon the wild
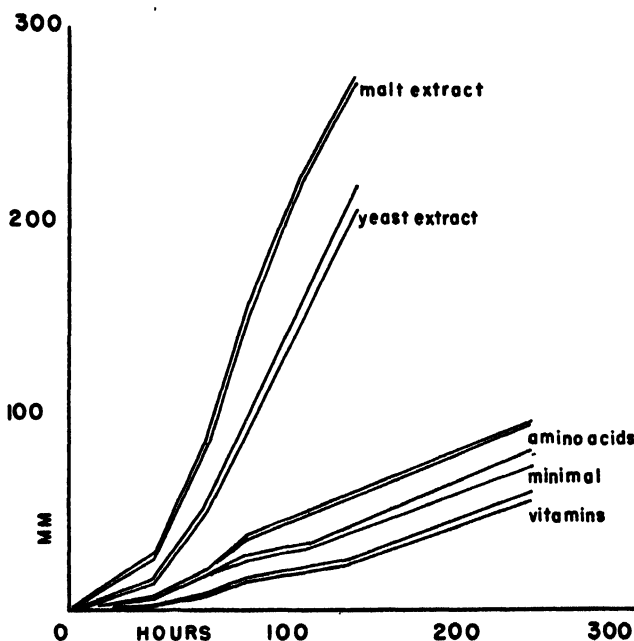
Figure 3. Growth of wild type *Neurospora* (strain E 5256A) at 30 C on solid minimal medium containing 10^{-3} moles per liter of indole and the various components of the complete medium indicated on the graph. These components were present in twice the amounts used in the *Neurospora* "complete" medium described by McElroy, Cushing, and Miller (1948).

type and occurred in the presence or absence of 10^{-3} moles of indole. Work is now in progress to determine the genetic relationship of these responses.

The work of Mitchell and Nyc (1948) on the synthesis of nicotinic acid suggested attempts to learn the nature of the specific antagonists of indole inhibition and the inhibitor of the mutant strain. These consisted of adding equimolar concentrations of available metabolites, known to be involved in this synthesis, to 10^{-3} M indole minimal medium. However, no reversal of inhibition was obtained by this method. Compounds tested included nicotinic acid, anthranilic acid, indole-3-acetic acid, DL-serine, and L-tryptophan. In addition, starch, dextrin, maltose, and glucose were also tested, as well as hydrolyzed casein, yeast nucleic acid, and the vitamin mixture in twice the amounts used in testing

for the occurrence of biochemical mutations (McElroy, Cushing, and Miller, 1948). Additional studies upon the properties of the indole antagonist are in progress. It may be of interest to note in this connection that certain adenine-requiring strains of *Neurospora* are inhibited by low concentrations of indole (Sein, Mitchell, and Houlahan, 1948).

The foregoing observations suggested that an antagonist similar to that found in malt and yeast extracts might be produced by the indole-resistant strains. However, experiments involving the growth of *Neurospora* upon autoclaved media prepared from culture filtrates of 20-31 and the wild type grown in the presence and absence of indole did not reveal the presence of heat-stable antagonists, and render it unlikely that this is the case. A search for possible heat-labile antagonists produced by this strain has yielded inconclusive results so far.

TABLE 3

Genotypes of ascospores obtained from a cross of indole-resistant temperature-sensitive strain 20-107 with wild type

ASCUS NUMBER	GENOTYPE OF ASCOSPORES			
	++	mt	+t	m+
A	1		1	
B	2	3		
C		2	2	2
D	1	1		
E			2	4
F		2	2	1
G				2
H	2	2		

++ = wild type morphology and temperature-resistant.
mt = mutant morphology and temperature-sensitive.
+t = wild type morphology and temperature-sensitive.
m+ = mutant morphology and temperature-resistant.

Mutagenic properties of indole. Indole resistance has only been observed to arise once in this study, this being in the original IA strain. Numerous controls consisting of wild type *Neurospora* growing on indole have never been observed to adapt, and an extensive series of subcultures have also yielded negative results. These observations make it virtually certain that the gene mutation involved in adaptive regulation of growth achieved by the IA strain arose by chance and was not specifically induced by the action of indole.

The possibility also exists of course that indole may act as a nonspecific chemical mutagen, and with this in mind classical tests for the presence of biochemical mutations were made upon all the isolates obtained from the original cross between IA and wild type strains. These tests were made possible by the fact that ascospores obtained from these crosses were isolated on complete medium, and the resulting strain could thus be tested upon minimal medium, this being done at 36 C. No attempt was made to go beyond a cursory exploration of this point

at the time. However, it is of interest that out of a total of 132 wild and mutant ascospores tested, representing 55 individual crosses, one temperature-sensitive mutant was obtained. This mutant (20-107), though morphologically indistinguishable from the indole-adapted mutants described earlier, would not grow at 36 C on complete, minimal, or indole media. Table 3 presents data showing that the peculiarities of this strain involve a mutation separate from that associated with enhanced ability to grow on indole. In this connection, it should be recorded that 20-31 has lost its ability to grow at 36 C on 10^{-3} indole and other media during a period of approximately 9 months since its isolation, and that 4 other mutant strains out of a total of 10 tested have indicated a similar physiological alteration. Although these observations are far from conclusive, they do suggest that the mutagenic properties of indole should be investigated, particularly as the alterations noted do not appear to have selective value in the presence of indole, and as the spontaneous occurrence of definite biochemical mutations appears to be a rare event in *Neurospora* (data on more than 3,000 untreated ascospores collected from various sources by the authors).

DISCUSSION

The observations reported here were made while investigating the possibility that specific gene mutations might be induced by using organic compounds capable of combining with specific genes and thus altering their reproductive capacities (Sturtevant, 1944; Emerson, 1944, 1945). It was hoped that the use of naturally occurring metabolites in abnormal amounts might favor observations upon the role of directed gene mutations as metabolic regulators. This point has recently been discussed by Monod (1947), who refers to the considerations of Wright and others on the subject.

Without elaboration of the ideas advanced by these authors, it may be said that the results of our study to date support the view that directed gene mutations are not involved in the regulative processes ordinarily carried on by cells; for, if this were the case, one would expect at the least that adaptive mutations would occur at a high rate in the presence of inhibitory concentrations of normal metabolites. Actually, such adaptations appear to be rarely achieved by *Neurospora*, and in the instance in which adaptation to indole toxicity occurred, the mutation involved appears to have arisen spontaneously. This conclusion is in agreement with the results of many other workers on mutations occurring in microorganisms (cf. Lederberg, 1948, for a review of the literature on this point), all of whom have failed to detect any cases of specific induction of mutations brought about by the substrate.

However, even though available evidence favors the view that substrate-directed mutations do not play a role in the regulative changes occurring in cells, it must be recalled that the connection between the specificity of gene action and the quantitative regulation of cell constituents is in a critical period of investigation. Such activities as the production of cellular antigens (Sonneborne, 1948; Kimball, 1947), antibodies, adaptive enzymes (Spiegelman, 1946; Stanier, 1947), virus particles (Price, 1948; Hershey and Rotman, 1949; Delbrück, 1948),

and other products that are made in varying ways by cells of the same genetic constitution are only just now coming under experimental control. In spite of this, it is already becoming apparent that the distinction of factors responsible for the alteration of the specific action of genes, as opposed to those affecting cytoplasmic activities, may eventually become more difficult to make along lines now drawn, at least in the case of some phases of genetic activity (cf. the discussions of the authors referred to above and Emerson and Cushing, 1946).

Lederberg (1948) has recently commented upon the value of microorganisms as material for the study of experimental evolution, particularly because large populations can be readily sifted to disclose new gene forms whose actions may be described in relatively precise terms. The present study suggests that, along these lines, one might hope to simulate some of the problems that must be solved by organisms, not only when they encounter antibiotics produced by other species, but whenever their own internal biochemical milieu changes in response to genetic alterations arising from the action of the forces influencing population genetics. (Consider for example the papers of Zalokar, 1948, and Emerson, 1948, on the sulfanilamide-requiring strain of *Neurospora*) That the evolutionary implications of such studies are not restricted to microorganisms is apparent when one considers the variety of potentially toxic compounds produced by or encountered by higher organisms (Gray and Bonner, 1948).

In conclusion it may be noted that the only other case known to the authors relating mutation to the toxicity of indole is that recorded by Delbrück (1948). Here it is reported that Anderson's viral strain T4 (one of two strains of phage requiring tryptophan for absorption on *Escherichia coli*) is inhibited in its action by minute amounts of indole (and to a somewhat lesser degree by skatole) and that this strain is capable of mutating to a type no longer requiring tryptophan and no longer inhibited by indole.

SUMMARY

The effect upon wild type *Neurospora crassa* of relatively high concentrations of normal metabolites was considered. Of the compounds studied p-aminobenzoic acid, tryptophan, anthranilic acid, and indole-3-acetic acid were found to be inhibitory in concentrations of 10^{-2} moles per liter or more. Indole was found to be inhibitory in concentrations of 10^{-3} moles per liter or more.

A strain of *Neurospora* showing enhanced ability to grow in the presence of 10^{-3} indole was investigated genetically, and this ability was associated with a mutation, the properties of which were also studied.

The mechanism of action of indole inhibition was studied, and an antagonist to this action was discovered in yeast and malt extract that has not yet been identified.

Other points associated with these phenomena are considered.

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THE ACTION OF CATIONIC DETERGENTS ON BACTERIA AND BACTERIAL ENZYMES[1]

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The bactericidal action of the quaternary ammonium detergents has not yet been satisfactorily explained. Recent reviewers (Putnam, 1948; Rahn and Van Eseltine, 1947; cf. Glassman, 1948) have not agreed that the amounts of these detergents lethal to bacteria are insufficient to cause any general denaturation of the bacterial proteins (Valko, 1946). Action by enzyme inactivation, either primary or secondary to some other injury, has been repeatedly suggested. Respiratory and glycolytic activity is depressed by detergents, and the inhibiting amounts were later shown to parallel roughly the lethal amounts (Baker et al., 1941a,b). However, the frequent survival of cells at concentrations of detergent which produced marked respiratory inhibition has cast doubt on an action by direct inhibition of enzymes. Hotchkiss (1946) demonstrated that nitrogen and phosphate compounds diffuse out of the cells with bactericidal amounts of detergents. As an alternative to the action by enzyme inhibition he suggested that the loss of substrates and cofactors by diffusion from the cytolyzed cells might account for the death of the cells and the observed metabolic inhibitions.

We have investigated the problem because objections can be made to much of the earlier work. Confirmation is apparently required that bactericidal amounts of detergents are well below those amounts causing general protein denaturation. The fact that *some* enzymes survive treatment with a bactericidal agent (Rahn and Schroeder, 1941; Greig and Hoogerheide, 1941; Bucca, 1943) has sometimes been interpreted as evidence against action by *specific* enzyme inhibition. More important is the lack of precise correlations between the lethal and the metabolic inhibitory amounts of detergent. Hotchkiss and others (Glassman, 1948) have pointed out that these irregularities may all be attributed to the comparison of quantitative metabolic measurements with the essentially qualitative determinations of viability by subculture. If cell death and metabolic inhibition are demonstrated to occur together with the same amount of detergent, an investigation limited to intact cells still leaves one unable to decide which of the results is cause and which effect (Roberts and Rahn, 1946). This decision would be simplified if the sensitivity of the enzymes in question, in cell-free form, to the detergents at the bactericidal levels was known.

METHODS

Escherichia coli was grown for 16 to 24 hours in 8-liter quantities of Difco casein digest broth, aerated by a stream of filtered air. Harvesting was accom-

plished with a Sharples supercentrifuge. The bacteria were washed in the centrifuge with 3 to 5 liters of distilled water and then suspended as a thick paste in water and stored at 0 C. All suspensions of *E. coli* used for manometric experiments were again washed with 10 volumes of water, centrifuged, and appropriately diluted according to the nitrogen content as determined by the micro-Kjeldahl method. Detergent concentrations were calculated from the percentage composition given by the manufacturers. In the manometric measurements of bacterial metabolism each cup contained 0.25 mg of bacterial protein N (1.5×10^{11} bacteria per mg N), 0.5 ml of 0.2 M phosphate buffer pH 7.0, and detergent and water to make a total volume of 2.5 ml. The substrate, 0.5 ml of 0.1 M solution, was tipped in from the side arm after 10 minutes' equilibration at 38 C, and readings were made for 1 hour. The center well contained NaOH except in the glycolysis experiments; in these experiments bicarbonate buffer was used with 95 per cent N_2 and 5 per cent CO_2 in the gas phase. Bacterial counts were made by diluting with sterile saline the contents of the manometric cups at the end of an experiment and inoculating aliquots into poured plates of Difco Endo agar. Counts were recorded after 24 hours' incubation. Each plate count was made in duplicate, and the average is expressed as a percentage of the average control count.

Arginine decarboxylase was prepared and measured under the conditions used by Gale and Epps (1944). The lactic acid oxidase was prepared by grinding the stock suspension of *E. coli* cells in the Booth-Green mill for 3 hours and centrifuging the resulting slurry after diluting it twofold with water. The cell-free supernatant, referred to as crude enzyme, is stable for over 1 week at 0 C and was used in most experiments. The oxygen uptake with the lactic acid oxidase was assayed for 10 minutes after tipping 0.5 ml of 0.2 M lactate from the side arm into 1.0 ml of enzyme and 1.3 ml of 0.05 M phosphate (pH 7.0) with and without the addition of detergent. With O_2 in the gas phase the activity under these conditions persists for 30 minutes and is proportional to the enzyme concentration between the limits of 10 and 140 mm^3 O_2 per 10 minutes. Pyruvate formation was determined with the salicylaldehyde method.

Measurement of the bactericidal amount of detergent. No one concentration of detergent was found to be generally bactericidal, contrary to the general assumption. As was found to be the case with chlorine (Knox *et al.*, 1948), a definite *amount* of detergent per *amount* of bacteria is reproducibly bactericidal. The lethal amount of detergent is therefore expressed as a bactericidal ratio, instead of a bactericidal concentration which would be meaningful only with given volumes of solution and numbers of bacteria. For convenience the bactericidal ratio is given as micrograms of detergent per mg of bacterial nitrogen, since in this form it is equally applicable to enzymes (per mg of protein nitrogen). That the inhibition of lactate oxidation by *E. coli* cells is proportional to the amount and not to the concentration of detergent is shown in table 1. The ratio of amounts of detergent and protein, and not the detergent concentration, as the determining factor may also be demonstrated from the experiments of previous workers in which sufficient data have been given. In the experiments of Sevag and Ross (1944), for example, a given amount of zephiran is lethal for only a certain

amount of yeast, and with the addition of serum still more zephiran is necessary, proportional to the amount of extraneous protein added. The precipitation of proteins, which occurs with much higher amounts of detergent, is also dependent upon the detergent-protein mass ratio (Glassman, 1948).

TABLE 1

Inhibition of the lactate oxidation by E. coli cells: proportionality to the detergent-bacteria ratio and not to the detergent concentration

BACTERIAL CONCENTRATION	ZEPHIRAN CONCENTRATION	ZEPHIRAN-BACTERIAL RATIO	PER CENT INHIBITION
mg N/ml	μg/ml	μg Z/mg N	
0.325	42	129	17
0.244	42	172	44
0.163	42	257	95
0.081	42	515	100
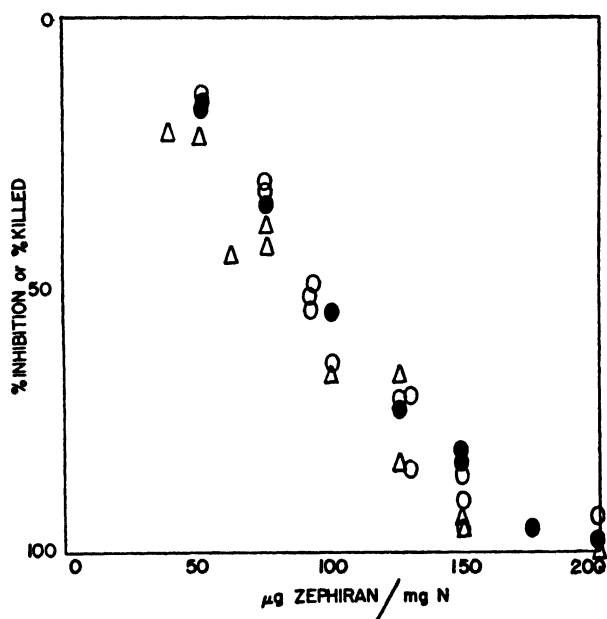
Figure 1. The amount of zephiran per mg of bacterial nitrogen to kill *E. coli* (△), to inhibit the glucose oxidation of *E. coli* (○), and to inhibit the lactic acid oxidase isolated from *E. coli* (●). Bacterial counts were made at the conclusion of the manometric determinations of glucose oxidation.

Comparison of the bactericidal effect of a detergent with its metabolic inhibition, and comparison of the bactericidal effects of different detergents, have been made at levels giving 50 per cent mortality and 50 per cent inhibition. The determinations of viable cells and of metabolic activity are comparable at this point, whereas serious errors are inherent in such comparisons at the point of 100 per cent sterilization.

Correlation of metabolic inhibition with bactericidal action. In figure 1 are given

the percentages of bacteria killed by increasing amounts of zephiran per mg of bacterial nitrogen. A second curve shows the per cent of inhibition of glucose oxidation by these cells, measured in the same experiment. The agreement, within our experimental error, that half the cells are dead and half the glucose oxidation inhibited at the same amount of detergent has been found in all instances with many different batches of bacteria. This same relationship was found to occur with a series of other cationic detergents. The amounts of these detergents which give 50 per cent killing and 50 per cent inhibition of glucose oxidation as determined from curves similar to that of figure 1 are given in table 2.

The metabolism of other substrates is not inhibited by a given amount of detergent to the same degree as is glucose oxidation (table 3). The higher amount necessary to inhibit lactate oxidation in the intact cell may perhaps be associated with the considerable stimulation of lactate oxidation observed in intact cells

TABLE 2

Comparison of the amounts of various detergents which kill E. coli, inhibit their oxidation of glucose, and inhibit the cell-free lactic acid oxidase

DETERGENT	50% KILLED	50% INHIBITION OF GLUCOSE OXIDATION	50% INHIBITION OF LACTIC ACID OXIDASE
	$\mu g/mg\ N$	$\mu g/mg\ N$	$\mu g/mg\ N$
1-*n*-Hexadecyl-pyridinium Cl (ceepryn)...........	40	27	31
(C8-18) Alkyldimethyl-benzyl-ammonium Cl (zephiran)...............	95	88	96
Cetyl-trimethyl-ammonium bromide..	120	110	107
N-(nonyl-naphthyl-methyl)-pyridinium chloride (emcol 888)...................	140	140	114
N-(lauryl-colamino-formyl-methyl)-pyridinium Cl (emulsept)...................................	260	225	169

with less than bactericidal amounts of detergents. This variation between the sensitivities of different oxidative and glycolytic reactions is not large. The inhibition of reactions such as that of arginine decarboxylase and of catalase (Roberts and Rahn, 1946), on the other hand, occurs only with a large amount of detergent (table 4). The large amount necessary to inhibit arginine decarboxylase is of the order able to denature the protein. In experiments with the cell-free enzyme it can indeed be seen that inhibition occurs only with denaturation and precipitation of the protein. The mass ratio of detergent to protein necessary for denaturation of this bacterial enzyme is of the same order required to denature other proteins (Chinard, 1948) and is obviously much higher than the bactericidal ratio.

The increase of arginine decarboxylase activity in the intact cell, which occurs with bactericidal and higher amounts of detergent, may be an expression of the increased cell permeability to the substrate, which occurs under these conditions. This effect has been recently used to measure the intracellular free amino acids

upon their diffusion out of the cell (Gale and Taylor, 1947). The increased activity of this enzyme, which requires the coenzyme pyridoxal phosphate, does not provide any support to the idea that the diffusion of coenzymes and substrates from the cell might account for the observed inhibition of other enzyme reactions.

TABLE 3

Amount of zephiran producing 50 per cent inhibition of various metabolic reactions of E. coli

REACTION	INTACT CELLS	CELL-FREE ENZYMES
	$\mu g/mg\ N$	$\mu g/mg\ N$
Glucose glycolysis...............................	125	—
Glucose oxidation..................	90	—
Pyruvate oxidation.................... .	115	—
Formate oxidation.......	135	—
Alanine oxidation.........	140	—
Lactate oxidation.....	180	96
Succinate oxidation.........	295	150
Arginine decarboxylation.......	*	5,400
Hexose diphosphate oxidation....	—	120
Hexose diphosphate glycolysis.......	—	1,000
Aldolase....	—	1,200

* Five thousand μg per mg N produced a 4-fold increase in arginin deecarboxylase activity (table 4).

TABLE 4

Effect of zephiran on arginine decarboxylase of E. coli

ZEPHIRAN	INTACT CELLS	ISOLATED ENZYME
$\mu g/mg\ N$	$Q_{CO_2}\ (mg\ N)$	$Q_{CO_2}\ (mg\ N)$
0	624	1,960
100	1,430	1,980
1,000	2,520	2,000 (+)
5,000	2,330	1,240 (++)
10,000	1,940	407 (+++)

+ = visible precipitation of the enzyme by detergent.

Enzyme inactivation as a mechanism for bactericidal action of detergents. It remains to be demonstrated that the detergents in bactericidal amounts can inhibit essential enzymes apart from any action on cell structure. Inspection of table 3, which contains those reactions known to be inhibited in the intact cell, does not reveal any one reaction the inhibition of which would account for all the results. Several enzymes, the reactions of which appeared to be inhibited in the cell by detergents, were therefore isolated to test their sensitivity to the detergents in the absence of cellular organization. The enzyme aldolase, as well as the whole glycolytic system producing acid and CO_2 from hexose diphosphate, were markedly resistant to detergents in the cell-free state, in

contrast to the glycolysis of glucose by the intact cell (table 3). The additional enzymes functioning in glycolysis in the intact cell, such as the phosphorylating enzymes, may be sensitive to detergents and account for this discrepancy, but such enzymes of *E. coli* have not yet been studied. However, two other systems, one oxidizing lactate and one oxidizing hexose diphosphate, could be prepared regularly in a soluble, cell-free form by the milling procedure used. Both of these isolated oxidizing systems were inhibited by bactericidal amounts of detergents (figure 1, tables 2 and 3). Both enzyme systems were unstable, the lactic acid oxidase perhaps less so, and it was selected for further study as a detergent-sensitive enzyme.

The Detergent-sensitive Lactic Acid Oxidase

The enzyme catalyzes the one-step oxidation of lactate to pyruvate (table 5) by molecular oxygen. It is therefore a lactic acid oxidase. It appears to differ from other lactic acid enzymes (dehydrogenases) previously described by reacting directly with oxygen. The lactic acid dehydrogenase from *E. coli* described briefly by Still (1941) was not tested with oxygen. Neither Still's enzyme nor the lactic acid oxidase require diphosphopyridinonucleotide (coenzyme I), and

TABLE 5

Oxidation of lactic acid to pyruvic acid by lactic acid oxidase

REACTION TIME	OXYGEN UPTAKE	PYRUVATE FORMED
	μ atoms	μ moles
40 min (1 ml enzyme)	31.2 ·	27.0
50 min (0.6 ml enzyme)	20.8	20.7
80 min (1 ml enzyme)	45.6	42.6

they are probably the same. The pH optimum of the enzyme is 7.0, with a marked decrease in activity below pH 6.0 or above pH 8.0. A relatively high concentration of lactate is required to saturate the enzyme, the Michaelis constant being approximately 0.0028 M under the assay conditions used (see "Methods"). Under these optimal conditions for assay, the crude enzyme maintains a linear uptake of oxygen amounting to about 30 mm^3 per mg protein nitrogen per 10 minutes for 30 minutes, after which the activity falls off rapidly. The enzyme reacts most rapidly with oxygen, and less rapidly with air, and this reaction is prevented by cyanide. Methylene blue can function as a hydrogen acceptor in the place of oxygen, and will in this way reverse the inhibition produced by cyanide.

The correspondence between the zephiran inhibition of the isolated lactic acid oxidase and the killing effect of zephiran on intact cells is shown in figure 1. Within the limits of error set by the techniques used, glucose oxidation in the intact cell, viability, and the cell-free lactic acid oxidase are all affected equally by this detergent. The effect of the detergent on the isolated enzyme, as on the intact cell, is dependent on the detergent-protein ratio and not simply on the concentration of detergent (table 6). The amounts of other detergents per mg of enzyme nitrogen which produce 50 per cent inhibition of the lactic acid oxidase

are given in the third column of table 2. The inhibitions of lactic acid oxidase by these detergents parallel for the most part their effects on viability and glucose oxidation, as is the case with zephiran. This would tend to confirm the view that the cationic detergents are bactericidal because they affect a specific enzyme system such as the lactic acid oxidase and not because they primarily alter the cell structure, since this enzyme still possesses, in the absence of the cell structure, the intact cell's pattern of sensitivity to detergents. Such enzymes inhibited by detergents may in turn be necessary to maintain the dynamic integrity of the cell membranes, and their inhibition would then account for the observed permeability phenomena and the cell death.

Mechanism of detergent inhibition of lactic acid oxidase. Further study of the lactic acid oxidase will be necessary to reveal the characteristic that produces the sensitivity of the enzyme, and therefore of the bacterial cell, to detergents. No added cofactors or coenzymes seem to be necessary for the reaction, since none of the known cofactors or coenzymes added increase the reaction, even after dialysis. Only threefold purification (by ammonium sulfate fractionation between 23 and 53 grams per cent) was possible due to the marked instability

TABLE 6

Proportionality of lactic acid oxidase inhibition to zephiran-protein ratio and not to the concentration of zephiran

ENZYME	ZEPHIRAN	ZEPHIRAN	OXYGEN UPTAKE	INHIBITION
ml	$\mu g/ml$	$\mu g/ml$ N	$mm^3/10\ min$	%
2.0	0	0	200	—
0.5	58	96	22	56
1.0	116	96	54	46
2.0	232	96	110	45

of the enzyme. Such purification as was achieved failed to reveal a need for cofactors or spectrophotometric evidence of a prosthetic group. In particular, the absorption curve of cytochrome b_2 recently described as part of the yeast lactic dehydrogenase was not found (Bach *et al.*, 1946). The reaction of lactic acid oxidase with oxygen and its inhibition by cyanide suggest the functions of a cytochrome component. Elimination of this cytochrome function by cyanide does not alter the detergent inhibition. Thus the anaerobic dehydrogenation reaction with the enzyme in the presence of methylene blue and cyanide shows the same sensitivity to detergents as does the reaction with oxygen. The cytochrome component, if it exists, is therefore not the site of detergent action. Enzyme which has been inhibited by detergent cannot be reactivated by removal of the detergent, by additional substrate, or by any known cofactors. There is no indication of prosthetic group separation or of protein denaturation in such an inhibited enzyme.

DISCUSSION

The most complete studies of inhibition of bacterial metabolism and bactericidal activity of surface-active agents (Baker *et al.*, 1941*a,b*) have shown a

general correlation of these two effects for a wide variety of detergents and for different bacterial species. However, the results, because of the methods used, do not support the hypothesis that a detergent-sensitive enzyme exists, the inhibition of which causes the metabolic depression and death of the cell. Although the two effects were determined in separate experiments and compared on the basis of detergent concentration instead of the bacteria-detergent mass ratio, identical conditions were maintained so that comparable bacteria-detergent ratios were achieved. In these experiments the occasional survival of cells with amounts of detergents which inhibited metabolic activity can then be attributed to the lack of precision of such comparisons at the point of 100 per cent sterilization. Metabolic measurements are essentially zero when 95 per cent of the cells in a suspension are inhibited, but subculture will still detect viable cells after more than 99.99 per cent of the cells are dead. The unusually large amount of detergent required to kill the last few cells, which may be protected by clumping (DuBois and Dibblee, 1946), heightens the discrepancy between these two methods. From these considerations, and the results reported here, a parallelism between the inhibition of glycolysis and certain sensitive oxidations and the bactericidal activity of the cationic detergents may be accepted. Observations were restricted to *E. coli* in the present instance to minimize secondary effects which might arise from the greater tendency of cells of other species to clump or lyse with detergents. The experiments quoted and others (Ordal and Borg, 1942) suggest that a similar parallelism will hold for other species. However, no study of the sensitivity of the appropriate cell-free enzymes of other species has been made.

The reported inability of even high amounts of detergent to inhibit bacterial metabolism more than 80 to 95 per cent (Baker *et al.*, 1941a,b) is no doubt referable to the high metabolic blanks of the cell suspensions used, and does not represent a persistence of the particular metabolic reaction under study. The cell suspensions used in the present study were washed immediately before use to remove the traces of other substrates from the medium and from disintegrated cells. Suspensions so treated have no measurable metabolism without added substrate and are fully inhibited with appropriate amounts of detergent.

It is clear from the information available that cationic detergents act in a specific manner on *E. coli* cells, and the cells of some other species, to produce concomitant inhibition of certain essential metabolic reactions, cell death, and an increase in the cell permeability. The experiments reported show that cell death (by milling, for example) is not necessarily followed by loss of enzyme activity, nor are enzyme reactions necessarily inhibited by an increased permeability (arginine decarboxylase). Primary inhibition of an essential enzyme reaction, on the other hand, might be expected to produce the organizational derangements that result in cell death and increased permeability. The demonstration that *E. coli* possesses presumably essential enzymes, which in cell-free form can be inhibited by the amount of detergent bactericidal for the intact cell, strongly suggests that the cationic detergents act by the specific inhibition of such enzymes.

The lactic acid oxidase is as yet insufficiently characterized to attempt any generalizations as to its relation with other detergent-sensitive enzymes of *E. coli*, or to the detergent-sensitive enzymes of other organisms. Sevag and Ross (1944) referred the action of zephiran on yeast to inhibition of the cytochrome c-cytochrome oxidase system. *E. coli* does not possess this familiar cytochrome system (Keilin and Harpley, 1941). Whatever cytochrome system is present in *E. coli* might well be present in the lactic acid oxidase complex (cf. the yeast cytochrome b_2-lactic dehydrogenase described by Bach *et al.*, 1946). Yet an action of the detergents on this moiety, as is assumed in the case of yeast, is apparently precluded in the *E. coli* lactic oxidase by the retention of detergent sensitivity even in the presence of cyanide.

SUMMARY

Several cationic detergents have been shown to kill *Escherichia coli* parallel with the inhibition of certain metabolic reactions of these cells. Other reactions persist in the presence of the lethal amounts of detergents. The detergents produce these effects of killing and inhibition proportional to the detergent-bacterial ratio, and not to the detergent concentration.

Escherichia coli possess certain enzymes which in cell-free form can be inhibited by the detergent-protein ratios which are bactericidal for the intact cells. One of these, the lactic acid oxidase, which does not require coenzyme I and which reacts directly with oxygen, is described. The specific inhibition of such detergent-sensitive enzymes can account for the metabolic inhibition, cell death, and increased permeability observed in bacteria with bactericidal amounts of cationic detergents.

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SOME FUNGOUS CONTAMINANTS OF OPTICAL INSTRUMENTS

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Surfaces of optical glass may be damaged from mold growth when the temperature is high and the relative humidity reaches, or exceeds, 80 for appreciable periods of time. Over the past ten years an effort was made to isolate and identify molds from damaged instruments, and eight species are reported here. Despite the small number, there is little overlapping with Hutchinson's (1946) list of molds. All branching forms on glass surfaces are not molds. One such "growth" on a glass surface was shown by microchemical methods to be crystallized potassium carbonate from a glass made with a potassium, rather than a soda, base. The etching of the glass surface was mainly due to the moisture associated with the mold leaching the surface, although organic acids and other chemicals excreted by the mycelium may also be involved (Jones, 1945).

When a surface appeared to have a mold growth, precautions were taken to culture it without contamination from local molds. Usually wiping the surface with a sterile cotton swab moistened with Difco nutrient or Sabouraud's agar would gather enough spores or mycelial fragments for inoculating a culture dish. When the contamination was resistant, it was removed with a sterile, heavy inoculating needle with about 5 mm of the end bent to a right angle. In some cases the fouling of the lens suggested the presence of mold (figures 1 to 3), but culturing failed to demonstrate any living molds. Figures 1, 2, and 5 are of the back lens of a 4-mm achromatic objective. Figure 3 is of a prism surface from a binocular, biobjective microscope, figures 4 and 8 of the upper inside surfaces of the objective lenses from the same microscope, and culturing yielded species of *Alternaria, Bassisosporium, Cladosporium,* and a pyrenomycete. The binocular microscope had been in the Canal Zone and Dr. Charles Thom noted a resemblance to molds received 20 years earlier from the same region.

A mold growth on a Leica camera lens (figure 7) was isolated and identified as *Pullalaris pullulans*. An ocular from a compound microscope used on an island near the Florida coast (figures 9 and 10) yielded on culture, *Monilia sitophola, Aspergillus niger,* and *Penicillium frequentans*. Growth around the cement and cork retaining ring of a polaroid polarizer of a polarimeter (figure 6) proved to be *Aspergillus niger*. I am greatly indebted to Drs. Kenneth B. Raper and Charles Thom (U. S. Department of Agriculture) for the identifications of the molds.

Although it did not seem very likely that optical glass alone would provide nutrient for mold growth, an attempt was made during the summers of 1942 and 1943 to grow these molds on six kinds of the more commonly used optical glasses. The surfaces were polished as they would be for use in instruments. The specimens were maintained in dishes above constant humidity mixtures for humidities

greater than 80 per cent, or with water to give a saturated atmosphere, for several months. These were at room temperature, which was within 84 to 95 F for a considerable portion of the summer. It was not possible to raise mold on the glass surfaces from heavy inoculations of spores by shaking them over the glasses in air, or from suspensions in drops of distilled water, nor did small amounts of
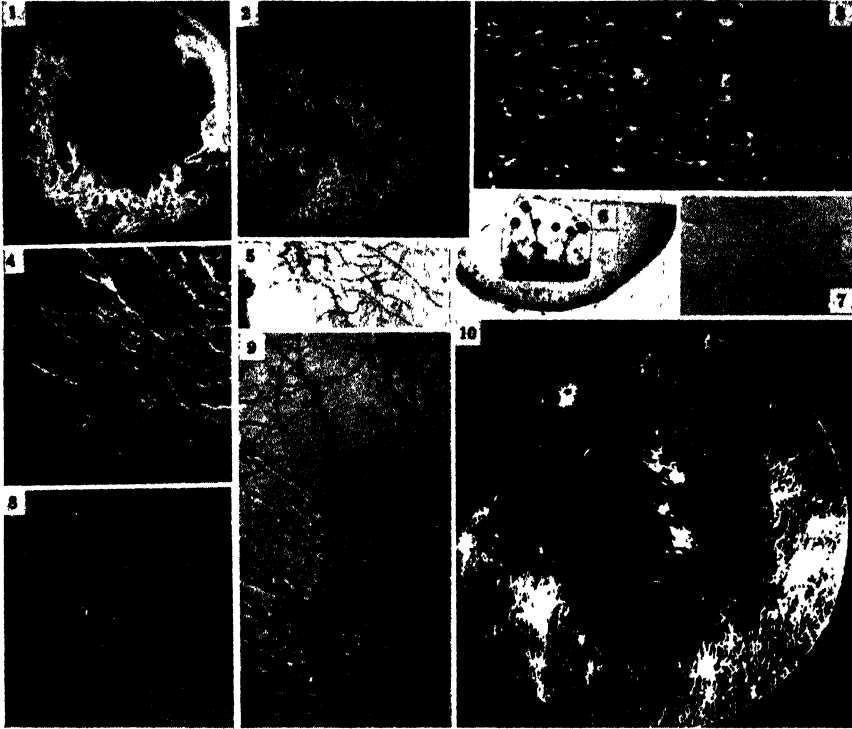
Figures 1-10

Figures 1 and 2. Outside surface of back lens of a 4-mm achromatic objective 5 ✕.

Figure 3. Etch on the surface of a prism of a binocular, biobjective microscope, probably due to mold growth. 20 ✕.

Figure 4. Mold filaments and moisture film of lens surface of figure 10. 20 ✕.

Figure 5. Enlarged portion of figure 2. 15 ✕.

Figure 6. Aspergillus growing around a polarizer. 2.5 ✕. Insert showing spore heads of an enlarged portion section. 8 ✕.

Figure 7. Pullularis growing on the surface of a camera lens. 20 ✕.

Figures 8 and 9. Enlarged portions of growth shown in figure 10. 25 ✕.

Figure 10. Top surface of the eye lens of a microscope ocular. 5 ✕.

mycelia transferred as free from agar as possible grow on the glasses. These results support the view that some organic matter, such as dirt or bodies of minute insects, must be present for mold to grow on glass surfaces and confirm the conclusions of Jones (1941) and Hutchinson (1946).

Meticulous cleanliness in manufacture and during the life of the instrument to keep sources of nutrition from the glass surfaces would prevent damage from

mold growth. Keeping the instruments in a cabinet or closet heated to a few degrees above room temperature will lessen the humidity and the growth of mold, especially if the instruments are to be stored for a vacation period. Cork retainers and similar materials may be mold-proofed by treating them with an alcoholic solution of phenylmercurihydroxide (Richards and Hawley, 1939). For periods of about two years, protection can be had by placing a capsule of a volatile fungicide within the instrument. Turner *et al.* (1946) recommend ethyl-mercurithiosalicylate, and Hutchinson (1947) reports cresatin (*m*-cresyl acetate) useful. Longer protection may be obtained from radium-containing foil placed around the lenses, as recommended by Vicklund (1943).

SUMMARY

The following molds were isolated and cultured from the glass surfaces of optical instruments: *Aspergillus niger* (obtained twice), *Monilia sitophola*, *Penicillium frequentens*, *Pullalaris pullulans*, and one species each of *Alternaria*, *Bassisosporium*, *Cladosporium*, and a pyrenomycete. Attempts to grow these molds on six of the commonly used types of optical glass without other nutrient matter failed. Unless the mold growth is removed from the glass surface, the moisture will damage the surface and leave it etched or raised where the mold growth occurred. Cleanliness, lowered humidity, and fungicides have been found useful protection from fouling of lenses by molds.

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THE SYNTHESIS OF AMINO ACIDS BY ESCHERICHIA COLI IN PURE CULTURES[1]

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The synthesis of a number of vitamin factors by intestinal bacteria, both *in vitro* and *in vivo*, has been well established. There is also definite evidence in ruminants and suggestive evidence in the rat and in man that a part, at least, of the daily requirement of these factors is furnished by this bacterial synthesis (Najjar and Barrett, 1945).

Some of the intestinal bacteria, *Escherichia coli* particularly, are capable of multiplying and producing their own cell substance in a medium consisting of an inorganic nitrogen source, salts, and glucose or some other simple carbon material (Koser, 1923). It seems possible, therefore, that these bacteria might synthesize additional quantities of amino acids in excess of their own cellular requirements and thus create a situation analogous to that of the vitamin factors. Or, conversely, the organisms might utilize considerable proportions of amino acids, made available to them, for their own growth and multiplication, and in this way deplete the growth medium of these factors.

Martin (1944) appears to have been the first worker to investigate the possibility of amino acid synthesis by bacteria in the intestinal tract of animals. His evidence, though inconclusive, suggests that the intestinal flora may contribute to the amino acid requirements of the rat. Hall and Sydenstricker (1947) reported a series of experiments on the production of methionine deficiency in the rat with low casein diets. They indicate, likewise, that on low protein diets the rat utilizes some of the amino acids elaborated by its intestinal microorganisms. Whether the source of these factors is the dead and disintegrated bacterial cells or free amino acids produced by the living cells in excess of their own requirements has not been shown.

Some work has been done by other investigators on the amino acid composition of *Escherichia coli* cells (Leach, 1905–1906; Eckstein and Soule, 1931; Camien, Salle, and Dunn, 1945; Stokes and Gunness, 1946; Freeland and Gale, 1947). Apparently, however, little or no attention has been given to the residual amino acid content of the media in which these organisms were grown. Evidence has been presented by Tatum and Bonner (1943) that tryptophan is synthesized from indole and serine by the ascomycete *Neurospora crassa*. These workers (1944) also made a limited number of experiments with *E. coli* and have reported that colorimetric tests indicated some synthesis of tryptophan from indole by

this organism, also in the presence of serine. Fildes (1945), however, was unable to confirm the work of Tatum and Bonner with *E. coli*, but thought that the activity of his strain of *E. coli* might have been greater in breaking down any tryptophan that was formed than the strain used by Tatum and Bonner. Fildes' tests with *Eberthella typhosa*, an organism that does not produce indole from tryptophan, were also negative, however. The investigation reported here includes the amino acid content of culture media after the cells had been removed, as well as the amino acid composition of the organisms themselves.

METHODS

Two strains of *E. coli*, one a laboratory stock strain and the other a freshly isolated strain from the hospital diagnostic laboratory, were grown in two types of media: first, an ammonium phosphate glucose medium, in which diammonium hydrogen phosphate was the sole source of nitrogen;[3] and second, a synthetic amino acid medium similar to that used for the microbiological assay of the amino acids, but with cystine omitted because preliminary tests had shown that this amino acid somewhat inhibited the growth of *E. coli*. Cultures were incubated at 37 C for 48 hours; following this they were autoclaved and the cells separated from the remaining culture medium by centrifugation. The cells were washed three times with cold saline and once with distilled water, evaporated to dryness on a water bath, and then dried overnight at about 105 C and weighed. The supernatant liquid and washings were combined, made up to a definite volume, and autoclaved at 120 C for 20 minutes to preclude further growth. The amino acids were assayed essentially according to the microbiological methods of Stokes, Gunness, Dwyer, and Caswell (1945), using *Streptococcus faecalis* (ATCC no. 9790) and *Lactobacillus delbrückii* (ATCC no. 9595). Proline and hydroxyproline were not obtainable during the course of these experiments; and since they had been shown by the authors of the method to be nonessential for these assay organisms, they were omitted from the basal medium. No adjustment was made for this omission because of the large safety factor present in the quantities of the other amino acids. Also, the final volume of the assay tubes was reduced from 10 ml to 4 ml with proportional reductions in all the additions.

RESULTS AND DISCUSSION

The amino acid composition of *E. coli* cells grown in the two culture media, the ammonium phosphate medium and the synthetic amino acid medium, is shown in table 1. The values shown in columns A and B represent individual cultures of the laboratory stock strain, and column C gives the values obtained with the freshly isolated strain. In general, the results obtained with the two different culture media and the different cultures of each are in fair agreement, most being within the limits of experimental error. The figures for arginine, histidine, leucine, phenylalanine, threonine, and valine agree within ±10 per cent. One value for isoleucine was higher than the other two, and one value for

[3] (NH₄)₂HPO₄, 1.5 g; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.2 g; NaCl, 0.1 g; glucose, 0.5 g; distilled water to 1.0 liter; pH adjusted to 7.0.

methionine was lower than the other two, and those of lysine and tryptophan are quite irregular. Unfortunately the yields obtained on the cells were not sufficient to make determinations for all 10 amino acids on each sample.

Also shown in table 1 are the results on *E. coli* reported by Stokes and Gunness (1946) and Camien, Salle, and Dunn (1945). Generally, the values obtained were somewhat lower than those reported by these other workers. This may be explained in part by the lower nitrogen values shown in the table, although recalculation to a basis of 16 per cent nitrogen does not completely eliminate the differences. Camien and his co-workers, as well as other investigators, have concluded that the amino acid composition of microorganisms is independent of the composition of the growth medium. Stokes and Gunness, however, obtained some

TABLE 1

Amino acid composition of Escherichia coli cells

(Per cent of dry weight)

CONSTITUENT	(NH₄)₂HPO₄ MEDIUM		AMINO ACID MEDIUM			STOKES AND GUNNESS*	CAMIEN ET AL.†	
	A	B	A	B	C		S₁	S₂
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Yield, dry wt./liter	115.50	81.00	110.00	138.50	92.00		530.00	190.00
Total nitrogen.....	10.58	10.63	10.30	12.00	11.95	13.19	13.38	13.08
Arginine...... ..	3.10	3.06	3.27	3.13	3.39	4.30		
Histidine...... ..	1.03	0.97		0.94	1.03	1.26	1.67	1.55
Isoleucine..		3.36	4.08	3.21		3.80	5.26	5.15
Leucine..	5.90		6.21			6.40	6.18	6.12
Lysine	3.22	2.22	4.08	2.88	3.32	4.50	5.27	4.82
Methionine		1.53		1.52	1.24	1.70		
Phenylalanine..		2.12		2.07		2.70		
Threonine..........		2.21		2.29		3.20		
Tryptophan........		0.33		0.52		0.79	0.50	0.57
Valine........... .	4.35		4.57		4.66	4.50	5.10	4.98

* Figures from Stokes and Gunness (1946).

† Figures recalculated to percentage of dry weight from Camien, Salle, and Dunn (1945).

differences in the amino acid content of *Penicillium notatum, Bacillus subtilis*, and *Streptomyces griseus* by varying the composition of the media and by aeration of the cultures. The results reported here indicate that not only is there some constancy of the amino acid composition of the cells in a single medium, but also there is little difference in the cells grown in the two types of media employed.

Attempts were made early in this work to assay the supernatants and washings of the ammonium phosphate medium cultures. These, however, proved too dilute, at least for histidine, even after 10-fold concentration on a water bath. The amino acid medium, on the other hand, proved more fruitful. The amino acid content of the combined supernatants and washings from the amino acid medium cultures is shown in table 2. Hydrolyzed as well as unhydrolyzed aliquots were assayed to determine whether there was any combined amino acid, either in cells which might not have been centrifuged out or present in the liquid as peptides.

Neither seemed to be the case. In general, the hydrolyzed values were lower than the unhydrolyzed, suggesting destruction during the hydrolysis. Such differences were most marked for methionine and valine, but occurred also in histidine, leucine, lysine, phenylalanine, and threonine. The values for arginine and isoleucine were irregular in this respect. It is probable that considerable tryptophan

TABLE 2

Amino acid content of the combined supernatants and washings from the amino acid medium cultures

CONSTITUENT	MICROGRAMS PER ML					
	A		B		C	
	Hydro.	Unhyd.	Hydro.	Unhyd.	Hydro.	Unhyd.
Total N	440.00		448.00		442.00	
Arginine		24.50* 27.54	6.80	6.64 5.93	20.80	21.88 24.50
Histidine			156.80	178.40		139.40
Isoleucine	83.00	69.00	77.20	97.50		
Leucine	106.50	115.40 127.50		125.70		136.45
Lysine		11.40 12.46	14.60	16.40 18.63	10.90	11.74 16.13
Methionine		110.00	80.20	110.00 109.40	69.20	102.50 109.50
Phenylalanine			77.00	87.00		
Threonine			67.95	75.50		
Tryptophan	135.00 135.75	172.00†	138.00 143.25	162.00†	137.03	164.00†
Valine	88.60	126.15		123.35	112.70	138.80 144.00

* Two values in a block indicate two individual determinations.

† These values were obtained from a later series of cultures.

was destroyed also, but the figures for unhydrolyzed tryptophan are not comparable, since they represent different preparations than do the hydrolyzed values.

Among the three lots of culture media, A, B, and C, comparison of the unhydrolyzed values in table 2 reveals fair agreement in the figures for leucine, lysine, methionine, tryptophan, and valine. Culture medium B was low in argi-

nine, and histidine and isoleucine appeared to be irregular with only two values for each.

Table 3 shows the total recoveries from the cells and from the unhydrolyzed supernatants of the three amino acid medium cultures, the combination of these two, and the percentage of the original quantity of the amino acids recovered. The most interesting results are those for valine, leucine, and methionine. The recoveries for these three amino acids are all greater than 100 per cent, indicating synthesis of these amino acids in excess of that originally present in the medium. The average percentage of recovery for valine was 138.5, for leucine 128.3, and for methionine 109.5. In contrast to this, arginine and lysine appear to have been utilized to a greater extent than any of the other acids assayed, or at least they have not been replaced in the media to the same degree. The average recovery for arginine was only 11.1 per cent and that for lysine 18.3 per cent. Histidine,

TABLE 3

Recoveries per liter of original amino acid medium

CONSTITUENT	ORIGINAL QUANTITY IN MEDIUM	CELLS	SUPERNATANT	CELLS PLUS SUPERNATANT	AVERAGE RECOVERY
	mg	*mg*	*mg*	*mg*	*%*
Total N	518.30	12.93	443.00	455.93	88.00
Arginine..	200.00	3.69	18.50	22.19	11.10
Histidine	200.00	1.13	158.90	160.03	80.00
Isoleucine...	100.00	4.48	83.25	87.73	87.70
Leucine....	100.00	6.84	121.45	128.29	128.30
Lysine... ..	100.00	3.85	14.46	18.31	18.30
Methionine. ..	100.00	1.62	107.85	109.47	109.50
Phenylalanine . ..	100.00	2.87	87.00	89.87	89.90
Threonine... . .	100.00	3.17	75.50	78.67	78.70
Tryptophan.. .	200.00	0.70	166.00*	166.70	83.30
Valine.. ...	100.00	4.67	133.78	138.45	138.50

* Obtained from a later series of cultures.

isoleucine, phenylalanine, threonine, and tryptophan show much less striking changes from the original quantities present.

It will be noted that the amount of any one amino acid contributed by the cells is only a small part of the total recovered, except perhaps in the totals for arginine and lysine. Thus the excesses of valine, leucine, and methionine are largely extracellular and represent unbound amino acids.

The low values for arginine and lysine suggested that these two amino acids might have been limiting factors in the synthesis of one or more of the acids assaying greater than 100 per cent. Accordingly, new lots of the amino acid medium were prepared, containing double quantities of arginine and lysine. The values for valine, leucine, methionine, and tryptophan were not significantly increased in these new lots. The arginine and lysine in the new medium were not utilized quite so completely on a percentage basis, but the absolute utilization was greater than before.

CONCLUSIONS

The data presented lend confirmation to the conclusions of other investigators that the amino acid composition of microorganisms is fairly constant for any one medium and one set of growth conditions. There appears, also, to be little difference in the amino acid composition of *Escherichia coli* cells grown in the two types of media employed. The results, however, vary sufficiently from those of other investigators, who used different media, to suggest that the composition of the medium may affect the amino acid composition of the cells.

It may also be concluded from these data that certain amino acids, namely, valine, leucine, and methionine, are synthesized by *E. coli*, at least under the conditions of these experiments, in amounts greater than are required for the cellular protein, and that these additional quantities are present extracellularly in the culture medium. Arginine and lysine appear to be utilized to a greater extent than any of the other amino acids assayed, or are synthesized to a lesser degree.

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THE SLOW RECOVERY OF BACTERIA FROM THE TOXIC EFFECTS OF PENICILLIN

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It was shown by Bigger (1944), Parker and Marsh (1946), and Parker and Luse (1948) that if staphylococci were exposed to lethal concentrations of penicillin *in vitro*, and if the drug was removed before all the organisms had been killed, the viable survivors did not then immediately resume multiplication. There followed instead a period of bacteriostasis during which the organisms, presumably recovering from the toxic effects of the drug, remained essentially constant in number. As will be shown in the present paper, this sublethal toxic effect of penicillin is not peculiar to staphylococci, but has been observed with every bacterial species studied so far. A number of factors have been found to modify both the toxic effect of the drug and the subsequent recovery of the damaged bacteria. Some implications with respect to the mode of action of penicillin are discussed in the text.

METHODS AND MATERIALS

Suspensions of bacteria during the logarithmic phase of growth were exposed at 37 C to varying concentrations of penicillin G. At intervals during this period, aliquot samples were withdrawn and the bactericidal action of the penicillin was terminated by the addition of "clarase" (Takamine) or "penicillinase" (Schenley) in appropriate concentrations. In the amounts used, the penicillin was almost completely inactivated within less than 5 minutes. This was shown by the fact that if mixtures of penicillin and the inactivating agent in the experimental concentrations were incubated for 5 minutes and then inoculated with bacteria, the organisms grew out at the same rate as they did in control tubes containing no penicillin.

The number of viable organisms[1] in the aliquot samples at the time of penicillin inactivation was determined by plating out serial 40-fold dilutions in an appropri-

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[1] It has been shown with cultured treponemata that organisms already rendered nonviable by penicillin may nevertheless remain actively motile for a number of hours (Eagle and Musselman, 1944). The agar plating technique here used determines, not the number of organisms that are still alive at the moment of penicillin inactivation, but the number of viable organisms capable of growing out in subculture. It follows that the bactericidal process as measured by this technique should stop abruptly when the penicillin is removed, unless the reacting mixture contains toxic factors other than penicillin which continue to cause the death of the organisms after the disappearance of the drug, but which are inactivated by dilution in blood agar. In most instances, the bactericidal process did cease abruptly on the addition of penicillinase; in some of the experiments, however, there was an unexplained continuing decrease in the number of viable bacteria for a period of approximately one hour.

ate medium also containing the penicillin inactivator. The various aliquot samples were then replaced in the incubator and were similarly subcultured at intervals to determine the rate of bacterial remultiplication. All the bacterial plate counts were made in duplicate, and the numbers and percentages given in the tables and figures are the averages of these duplicate determinations. The difference between the two duplicates averaged approximately 25 per cent. Thus, in the 25 determinations of table 4, the difference between the duplicate determinations varied

TABLE 1

Illustration of the slow recovery of β-hemolytic streptococci (group A, C-203 strain) from the toxic effects of penicillin

The organisms (496,000 colonies per ml in final mixture) were exposed to concentrations of 0.1 (100) μg of penicillin G per ml in 2 per cent rabbit blood beef heart infusion glucose broth. At the time intervals indicated in the table, 0.5 ml of Schenley penicillinase S at 200 (2,000) "units" per ml were added to a 9.5-ml portion, giving a final concentration of 10 "units" in the tube containing 100 μg per ml. (The numbers of organisms in the table have not been corrected for the 5 per cent error introduced by the change in volume.) The tube was then incubated at 37 C. At intervals thereafter, 0.3-ml samples were withdrawn and added in duplicate to 11.7 ml of rabbit blood beef heart infusion glucose agar at 50 C.* Three to 5 such serial 40-fold dilutions were prepared from each sample, depending on the anticipated number of bacteria, and plates poured from all the dilutions. The number of organisms in the plate, and thus the total number in the original mixture, was determined after 48 hours' incubation, using as the indicator plate the one containing 10 to 400 colonies. The figures given in the body of the table are the percentage of viable survivors, referred to the original inoculum as 100. Each value is the average of the 2 duplicate determinations. The numbers in boldface type represent counts during the "static" period of recovery.

CONC. OF PENICILLIN, MICROGRAMS PER ML	TIME (HR) OF EXPOSURE TO PENICILLIN, BEFORE THE ADDITION OF PENICILLINASE	PERCENTAGE† OF ORGANISMS SURVIVING AND VIABLE AT INDICATED TIME AFTER INACTIVATION OF PENICILLIN											RECOVERY PERIOD HR‡
		0	¼	½	1	1½	2	2½	3	4	5	6	
0	—			230	860		4750	·		21,450			
0§	—		125	175	645		3350			18,050			
0.1	¼	**90**	80		87	120	240	295	920	2,540	5100	—	1.25
	1	5.4			8.0		7.5	6.5	13 4	36.5	185	510	2.5
	4	**0.45**		**0.43**	**0.43**	**0.4**	**0.24**	**0.43**	0.64	1.4	7.6	35.5	3
100	4	**0.44**		**0.4**	**0.36**	0.63	**0.28**		0.48	0.9	3.45	9.9	3.5

* Containing 0.017 per cent of Takamine "clarase" to inactivate possible traces of residual penicillin. (The stock 10 per cent solution of clarase was clarified by centrifugation and sterilized by filtration through a pyrex glass filter.)

† Referred to original inoculum of 496,000 per ml as 100.

‡ Obtained by graphic interpolation (cf. figures).

§ A control consisting of a tube originally containing 100 micrograms per ml, incubated at 37 C with the usual amount of penicillinase (100 units per ml) for just 5 minutes prior to inoculation. As here shown, the organisms grew out at almost exactly the same rate in this mixture as in the control tube containing only broth, indicating the essentially total inactivation of penicillin.

from 0 to 49 per cent of the larger value, averaging 15 per cent. One could estimate graphically (cf. figures) the period for which the bacteria had remained static in number and the point in time at which they had resumed multiplication to a significant degree.

EXPERIMENTAL RESULTS

The recovery of bacteria from the toxic effects of penicillin in vitro. A typical experiment with a group A β-hemolytic *Streptococcus* (strain C-203) is summarized in detail in table 1. As is there shown, when the bactericidal action of penicillin

was abruptly terminated by the addition of penicillinase, the number of viable organisms as determined by plate counts remained constant for several hours, during which the organisms were recovering from the toxic effects of the drug, and after which they resumed multiplication at a normal rate.

A number of similar experiments with 9 strains of 7 different bacterial species are summarized in table 2. Depending on the particular bacterial strain, and a number of variables to be discussed presently, the duration of the recovery period varied from as little as 0.3 hours to a maximum of more than 8 hours. Although the recovery periods as given in that table imply a simultaneous resumption of multiplication by all the bacteria, it is clear from the figures that in some of the experiments the transition from complete bacteriostasis to a normal rate of multiplication was gradual. In some of the experiments, also, the toxic effect of penicillin on the bacteria was manifested, not by a complete cessation of multiplication, but merely by a temporarily reduced rate of multiplication. It is not clear whether in such cases the reduced rate of multiplication resulted from a toxic process that had affected all the bacteria to essentially the same degree, or whether some of the organisms had undergone complete bacteriostasis while others were multiplying at a normal rate. Usually, however, the temporary bacteriostasis was complete, the number of organisms as judged by plate counts remaining constant within the limits of experimental error.

As is shown in table 2 and graphically illustrated in figures 1 to 3, the duration of the recovery period for a particular bacterial strain was affected to an important degree by two factors: the concentration of penicillin to which the organisms had previously been exposed and the duration of that exposure.

The effect of the time for which bacteria had been exposed to penicillin on the duration of the following recovery period. In the case of the C-203 strain of group A *Streptococcus*, penicillin at the maximally effective concentration of approximately 0.1 μg per ml exerted a toxic effect within 5 minutes and before any significant number of organisms had been killed. When the penicillin was then inactivated, the number of bacteria remained static for a period of 0.7 hours, after which they began to multiply at their normal rate (cf. figure 1). With a longer preliminary exposure to penicillin, the duration of the recovery period at first increased progressively, reaching a value of approximately 2.5 to 3 hours after 30 minutes' exposure. Thereafter, however, the duration of the recovery period was only slightly increased with more prolonged exposure. When the organisms were incubated with penicillin for 4 hours, the few organisms then surviving recovered and resumed multiplication within 2.5 hours after the removal of the drug. The top section of figure 3 summarizes the results with this and 3 other species (*Diplococcus pneumoniae* type I, *Staphylococcus albus*, and *Streptococcus faecalis* (strain H), with all of which the recovery period quickly rose to a maximum value and did not increase significantly thereafter. In several of the experiments there was an indication that the recovery period had actually decreased on prolonged incubation with penicillin (cf. table 2).   ·

With the other 5 bacterial strains here studied, however, the duration of the recovery period increased progressively with the time for which they had pre-

TABLE 2

The recovery of bacteria from the toxic effects of penicillin

(Summary of all experiments, with 9 bacterial species)

ORGANISM STUDIED, SPECIES AND STRAIN	CONC. OF PENICILLIN TO WHICH BACTERIA WERE EXPOSED (μg/ml)	TIME REQUIRED FOR BACTERIA TO RECOVER AND RESUME MULTIPLICATION AFTER PRELIMINARY EXPOSURE TO PENICILLIN FOR												
		Minutes					Hours							
		1	5	15	30	45	1	1½	2	3	4	6	8	24
Diplococcus pneumoniae type I	0.1		0.4,1	0.3		2		2.0,2.8	2		2.5	3.6		
	100							3.3			2.5	2.7,3.6		
Streptococcus pyogenes (C-203)	0.008	0		0				0.75			3.5			
	0.1–0.128	0.35,0.7		1.3,1.3	2.7,2.7		1	3.1,3.5	2.1		2.5			
	100	0.7		1.3	3±		2.5	3.6	2.7		3.3			3.2,6±
Streptococcus pyogenes (M-27)	0.024					1.4,1.8±		2.2±,3.7		2.3				6±
	256					2.8								6.9,7
β-Hemolytic Streptococcus, group B	0.1			0.65		2.1,2.4		2.4,3.5		3.25				4.6,>5
	0.15							3.5						5.6,>6
	256–2048			0.4		1.1		1.5,3.2		2±,3.3	3.8	3.5		
α-Hemolytic Streptococcus	0.1–0.15					0		1.3		1.5±		2.5		5.8,>6
	256					0						1.5		4.6,6.5,>6
Streptococcus faecalis (H)	8		0.3±	0.9±		1.9	1.6	1.75,1.8		2		1.6,2.3		0.55,1
	256–512		0.4±			1.		1.2,1.55		1.7		1.8,2		1.5,3,3.2
Streptococcus faecalis (M-3)	12			0.		0.8		1.75		2.2		2.7		4.3,4.3
	256			0.9						2.15		2.7		4.6
Staphylococcus aureus (Smith)	0.1		0.5	.6			0.3±,0.7		1.7		2	1.7	2.5	4±
	256		0.5,1.0				3,3.3		3,>4		6,6			6.4,>6,8,>8
Staphylococcus albus (M-14)	0.15			0		1.5		2.6		3		3.6		2.3
	256					1.9				3.5±		3±		2.8

viously been exposed to penicillin, within the time limits of the experiment. Figure 2 describes a single experiment with a group B hemolytic *Streptococcus*, in which the time required for the surviving bacteria to recover from the toxic effects of penicillin increased progressively from 1.1 to 5 to 6 hours as the preliminary incubation with penicillin was prolonged from 45 minutes to 24 hours. The lower section of figure 3 summarizes the results obtained in a number of experiments with this and 3 other strains which showed a similar preliminary progressive increase in the duration of the recovery period as the bacteria were
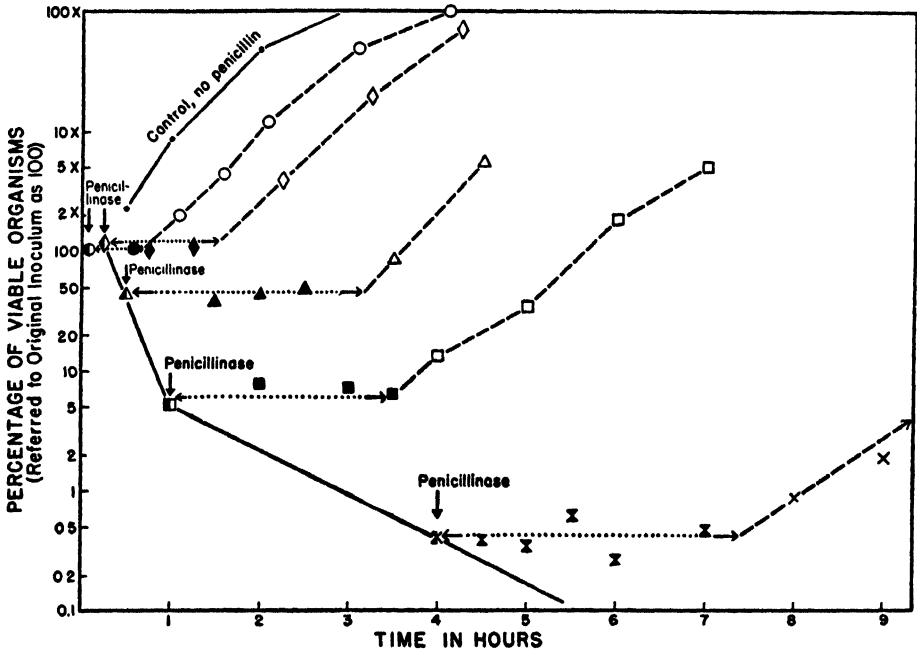
Figure 1. The effect of the time for which bacteria have been exposed to penicillin on the duration of the following recovery period. The experiment was conducted with a group A β-hemolytic *Streptococcus* (strain C-203) with which the recovery period reached essentially its maximum value after 30 minutes' exposure to the drug. The concentration of penicillin was constant (0.1 μg per ml), and the duration of exposure was varied.

incubated with penicillin for a longer and longer time. Each point plotted in the figure is the average of the individual recovery times listed in table 2.

The effect of the concentration of penicillin on the rate and extent of the bacterial damage. The toxic effect of the penicillin under present discussion was observed only at concentrations that were ultimately lethal to the organisms; and measured by the time required for the bacteria to recover and resume multiplication, the toxic effect usually developed most rapidly at those concentrations which were most rapidly bactericidal. Thus, with the C-203 strain of β-hemolytic *Streptococcus*, the most rapidly bactericidal concentration had been found to be on the order of 0.1 μg per ml; while concentrations of 0.008 μg of penicillin, although eventually lethal, killed the organisms much more slowly (Eagle and Musselman,

1948). Similarly, 0.1 μg per ml was maximally effective with respect to the rate at which the bacteriostatic effect became manifest, while concentrations of 0.008 μg per ml only slowly effected a bacteriostasis which persisted after the removal of the drug (cf. table 2 and figure 3). At the latter threshold concentration of penicillin, it required approximately 1 hour to produce a demonstrable toxic effect instead of less than 5 minutes at a concentration of 0.1 μg per ml; and the maximum sublethal damage to the organism, reflected in a recovery period of 2.5 to 3.5 hours, developed only after more than 2 hours' exposure instead of 30 minutes'.
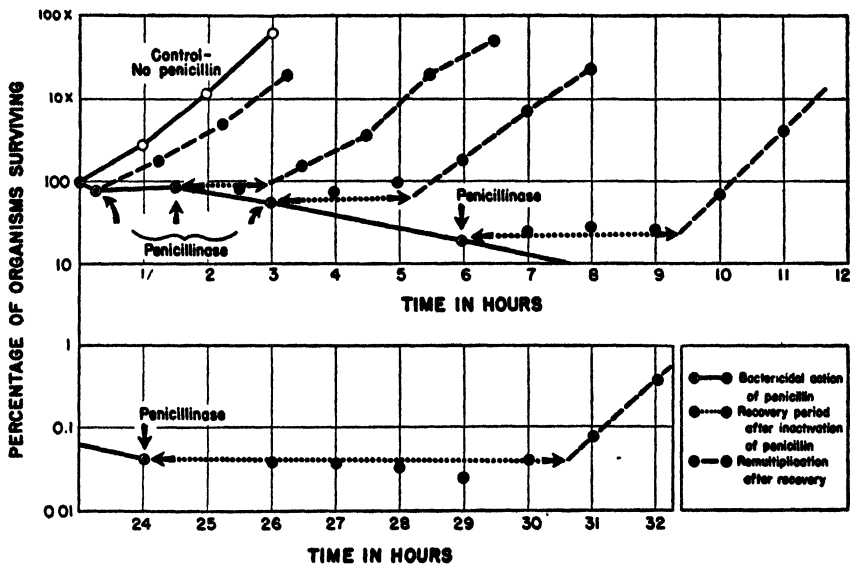
Figure 2. The effect of the time for which bacteria have been exposed to penicillin on the duration of the following recovery period. The experiment was conducted with a group B β-hemolytic *Streptococcus* (concentration, 256 μg per ml) that was only slowly killed by penicillin. With this organism the time required for the bacteria to recover from the toxic effects of penicillin increased progressively with the time for which it had been previously exposed to the drug.

The effect of very high concentrations of penicillin is also of interest. In the case of its bactericidal action, beyond a certain limiting concentration, even a 10,000-fold increase did not further accelerate the rate of bacterial death, and in some instances actually retarded that effect (Eagle and Musselman, 1948). With 8 of the 9 strains here studied, a similar relationship was observed in the sublethal bacteriostatic action of penicillin. In 7 of these 8 strains the toxic effect developed at the same rate whether the bacteria were exposed to, e.g., 0.15 to 256 μg per ml (cf. legend to figure 3); and with the 8th strain, a group B *Streptococcus*, there was a suggestion that high concentrations were even less rapidly toxic than low (cf. table 2). A paradoxical result was, however, obtained with the *Staphylococcus aureus* (Smith). This is shown in the curves marked by the filled and the open triangles in the lower section of figure 3. With this organism, exposure to 256 μg

per ml caused more damage (i.e., a more prolonged recovery period) than did similar exposure to a concentration of 0.1 μg per ml, this despite the fact that the lower concentration was much more rapidly bactericidal (Eagle and Musselman, 1948).
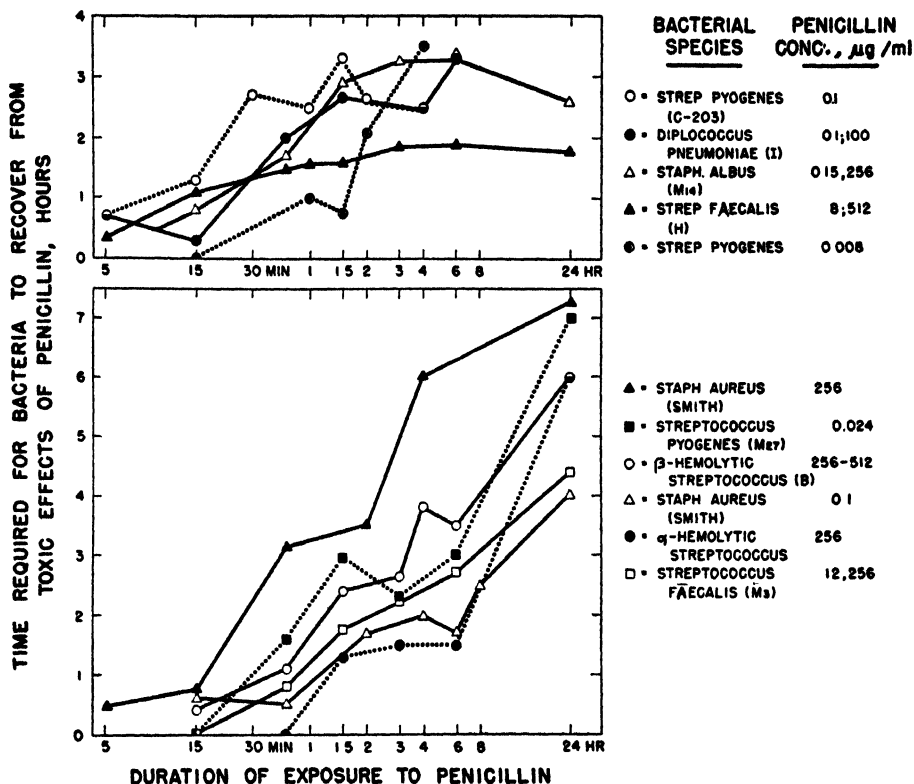
Figure 3. The effect of the time for which bacteria have been exposed to penicillin on the duration of the following recovery period: summary of all the experiments listed in table 2. Each point in the figure is the average of the individual recovery times listed in table 2. Section A of the figure includes the 4 strains with which the duration of the postpenicillin recovery period reached its maximum value after three-fourths to one and a half hours' exposure and did not significantly increase thereafter. Section B includes the 5 strains with which the duration of the recovery period increased progressively with the duration of the preceding exposure to the drug.

The relationships between the bacteriostatic and bactericidal actions of penicillin. With all 9 strains here studied, the bacteriostatic action of penicillin preceded its bactericidal action. In table 3 those strains have been arranged according to the time at which the bacteriostatic effect became evident (column 2). In each instance the organisms had stopped multiplying and the necessity for a postpenicillin recovery period of at least one hour had been established before a significant proportion of the cells had been killed (cf. column 3, table 3). Soon afterward, however, the bacteria began to die, so that, at the next time period tested, the

TABLE 3

Correlations between the bacteriostatic and bactericidal actions of penicillin

| | (1) CONC. OF PENICILLIN TO WHICH ORGANISMS WERE EXPOSED | (2) TIME REQUIRED FOR BACTERIOSTATIC ACTION OF PENICILLIN TO BECOME ESTABLISHED* | (3) PERCENTAGE OF ORGANISMS STILL VIABLE AT TIME INDICATED IN COLUMN 2 | (4) PERCENTAGE OF ORGANISMS VIABLE AT NEXT TIME PERIOD TESTED | | (5) AVERAGE TIME REQUIRED TO KILL 99.9 PER CENT OF BACTERIA | (6) BACTERICIDAL CONC. OF PENICILLIN | | (7) MAXIMUM RECOVERY PERIOD OBSERVED AT CONCENTRATIONS OF COLUMN 1 |
BACTERIAL STRAIN	$\mu g/ml$			Time in hr	%	hr	Minimal $\mu g/ml$	Maximally effective $\mu g/ml$	hr
Streptococcus pyogenes (C-203)	0.1	<15 min	100,119	¾ / 1	33,49 / 5.4	2-4	0.004	0.032-0.064	3.6
Diplococcus pneumoniae type I	0.1	>15 min < 45 min	100 / 63	1½	0.8	2-5	0.024	0.064-0.128	3.6
β-Hemolytic Streptococcus, group B	0.1	>15 min < 45 min	100 / 125,110	1½ / 3 / 6	11,39,56 / 6.3 / 10,23	5-10	0.016	0.064	3.3
Staphylococcus albus	0.15	>15 min < 45 min	150 / 20	3	0.05	Inconsistent values ?	0.016	0.064-0.128	3.6
Streptococcus pyogenes (M-27)	0.024	< 45 min	120,200	1½ / 3	24,43 / 0.1,0.6	3-9	0.004-0.008	0.016-0.032	6 ±
Staphylococcus aureus (Smith)	256	< 15 min > 60 min	114,160 / 76,110	2	5.5,8	17-21	0.016-0.032	0.064-0.128	8
Streptococcus faecalis (H)	256	45 min	93	1½ / 6	50 / 1.7	17-30	2-3	6-8	3.2
β-Hemolytic Streptococcus (group B)	256	45 min ±	80	1½ / 3 / 6	82,85 / 56 / 19	17-23	0.016	0.064	6
Streptococcus pyogenes (C-203)	0.008	1-1½ hr	42,72	2 / 4	2.1 / 0.8	10 ±	0.004	0.032-0.064	6 ±

Staphylococcus aureus (Smith)	0.1	> 1 hr < 2 hr	112, 140 11.5	4 6	1.8 0.11	5-12	0.016-0.032 / 0.064-0.128	4 ±
α-Hemolytic Streptococcus	256	> 45 min < 1½ hr	125 110	3 6	23 2.8	15-30	0.016-0.032 / 0.128	6
Streptococcus faecalis (M-3)	12	> 45 min < 1½ hr	126 84	6 24	73 28,33	> 48	2-3 / 6-8	4.3

* From data of table 2.
† From Eagle and Musselman, 1948.

proportion of viable organisms had fallen to a fraction of the original value (cf. column 4). Thus, when a strain of *Diplococcus pneumoniae* type I was incubated with 0.1 μg per ml of penicillin, it required between 15 and 45 minutes' exposure to damage the bacteria to the degree that, after the removal of penicillin, they resumed multiplication only after a recovery period of 1 hour. After 15 minutes' exposure all the organisms were viable; and after 45 minutes' exposure, when the bacteriostatic effect had been fully established, the proportion of viable organisms was still 63 per cent. On further incubation with penicillin, however, they began dying rapidly so that after 1½ hours' exposure the number of viable organisms had fallen to 0.8 per cent.

In the present experiments, the speed with which the toxic bacteriostatic effect became manifest was related to the rate at which the organisms were eventually killed by penicillin. Column 5 of table 3 gives the times required to kill 99.9 per cent of each of the bacterial strains here studied, based on previously reported studies (Eagle and Musselman, 1948) and additional subsequent experiments. With the exception only of the anomalous *Staphylococcus aureus* strain, there was a definite relationship between the rate at which the bacteriostatic action became manifest and the rate at which the organisms were ultimately killed by penicillin: the longer the time required to establish a lasting bacteriostatic effect (column 2), the longer, generally, was the time required to kill, e.g., 99.9 per cent of the bacteria (column 5).

In contrast to the findings of Parker and Luse with staphylococcal strains, in the seven species here studied there was no correlation between the "sensitivity" of the organisms to penicillin, i.e., the concentrations necessary to kill (column 6), and either (a) the rate at which the bacteriostatic effect developed, or (b) the maximum duration of the recovery period after the removal of penicillin. The concentration of penicillin that killed the bacteria at a maximal rate varied from 0.016 to 0.032 micrograms per ml for the M-27 strain of *Streptococcus pyogenes*, to 8 micrograms per ml for the 2 strains of *Streptococcus faecalis* (column 6 of table 3). The time required for the bacteriostatic effect to become established at the optimal concentration of penicillin varied from <15 minutes to 1½ hours (column 2), and the maximum duration of the recovery period varied from 3.2 to >8 hours (column 7). As is seen in table 3, neither of the latter variables bore any demonstrable relationship to the effective concentrations of penicillin.

The effect of environmental conditions on the toxic (bacteriostatic) action of penicillin and on the recovery of the organisms from that toxic effect. In order for penicillin to exert its toxic bacteriostatic effect, it is necessary that the organisms be in a favorable environment which would permit the growth and multiplication of the organisms were it not for the presence of the drug. (a) As is shown in figure 4, when pneumococci were exposed to penicillin at low temperatures (2 to 4 C), they were not poisoned by the drug and, on its removal, resumed remultiplication as rapidly as control suspensions similarly incubated without penicillin. (b) When a rapidly growing suspension of *Staphylococcus aureus* was concentrated by centrifugation so that multiplication stopped because of that concentration and was then incubated with penicillin for 4 hours, no significant proportion of the bacteria
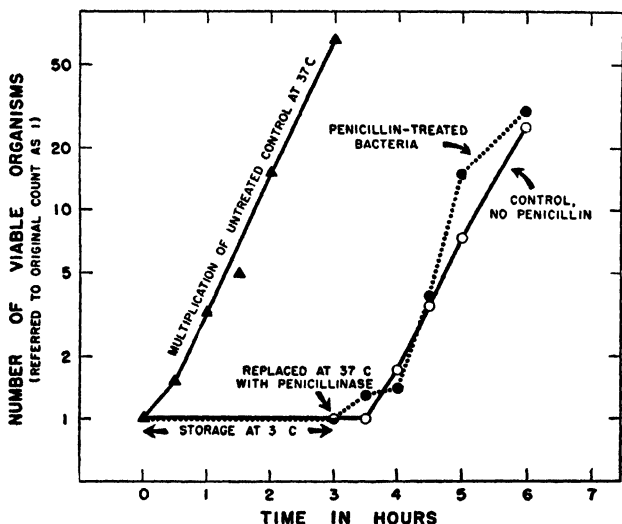
Figure 4. Absence of toxic (bacteriostatic) penicillin effect on *Diplococcus pneumoniae* (type I) at 3 C. A young (2-hour) culture was diluted 1:200 in cold blood broth containing 0.1 microgram of penicillin G per ml. The initial number of bacterial clumps (determined by plate count) was 155,000 per ml. After 3 hours at 3 C, penicillinase was added to a final concentration of 10 units per ml, and the tube was then placed at 37 C. As shown in the figure, the treated organisms grew out as promptly as a similarly treated control suspension containing no penicillin, without the preliminary recovery period characteristic of penicillin-damaged bacteria.
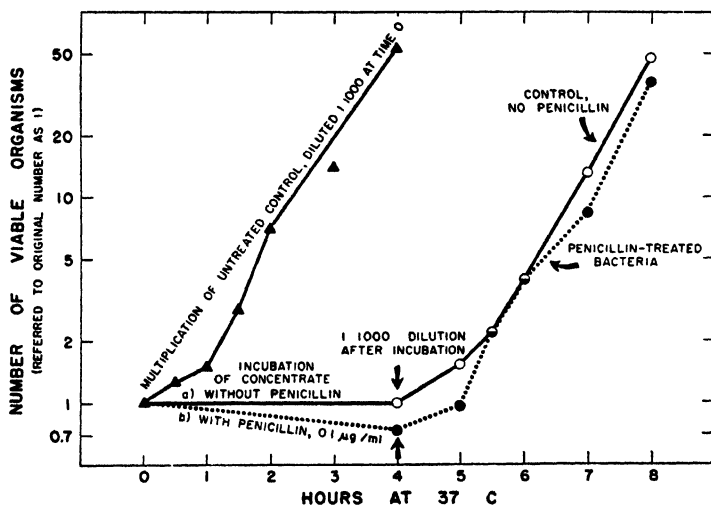
Figure 5. Absence of toxic (bacteriostatic) penicillin effect on concentrated suspensions of *Staphylococcus aureus* (Smith). A 3-hour culture in 2 per cent serum broth was concentrated 10-fold by centrifugation and penicillin G added to a final concentration of 0.1 microgram per ml. The initial number of bacterial clumps determined by plate count was 2.6×10^9 per ml. After 4 hours at 37 C the suspension was diluted 1:1,000 in warm broth containing penicillinase (2 "units" per ml) and incubated at 37 C. As shown in the figure, the penicillin-treated organisms grew out as rapidly as a similarly handled control suspension containing no penicillin, without the preliminary recovery period characteristic of penicillin-damaged bacteria.

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were killed; and when the suspension was then diluted, the organisms grew out without the delay characteristic of penicillin-damaged bacteria and as rapidly as a control suspension similarly incubated (figure 5).

Not only does the toxic effect of penicillin require conditions favorable to growth, but organisms already damaged by exposure to the drug can recover only

TABLE 4

The failure of bacteria to recover from the toxic effects of penicillin when stored at low temperatures (3 C)

A suspension of *Diplococcus pneumoniae* type I (5×10^6 ml) was incubated with penicillin G at 0.1 microgram per ml for 2 hours at 37 C. At that time, when 0.34 per cent were still viable, penicillinase was added to a final concentration of 10 "units" per ml, and aliquot portions were placed at (a) 37 C and (b) 3 C. Tube (a) at 37 C was subcultured at hourly intervals to demonstrate the recovery and remultiplication of the survivors. Tube (b) was kept at 3 C for 24 hours, when a portion was placed at 37 C, and plate counts made at hourly intervals. This was repeated with the remainder after 48 hours at 3 C. As a control, a 1:100 dilution of the original untreated suspension was kept at 3 C for 24 and 48 hours, and then placed at 37 C to determine the rate of multiplication. The results in the table are expressed as the percentage of viable bacterial clumps, determined by plate count, and referred to the original suspension as 100.

PREVIOUS TREATMENT OF THE BACTERIAL SUSPENSION	PERCENTAGE OF VIABLE ORGANISMS AFTER INCUBATION AT 37 C FOR						TIME (HOURS) BEFORE BACTERIA RECOVERED FROM THE TOXIC EFFECTS OF PENICILLIN AND RESUMED MULTIPLICATION
	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	
Exposed to 0.1 μg/ml of penicillin at 37 C for 2 hr, when penicillinase was added							
No further treatment......... .	0.34	0.21	0.4		8.3	38.3	2.2 ±
3 C—24 hr................... .	0.25	0.24	0.26	0.93	4.2		2.2
3 C—48 hr................... ...	0.3	0.21	0.28	0.88	3.3		1.9
Control suspension not treated with penicillin, but diluted 1:100 and then placed at 3 C							
3 C—24 hr.....................	0.98	1.59	10.8	27	203		< 1
3 C—48 hr.....................	0.88	1.64	7	21	155		< 1

Conclusion: Bacteria previously exposed to penicillin, and requiring approximately 2 hours at 37 C to recover from the toxic effects of that exposure, had not recovered appreciably after storage at 3 C for 48 hours, the subsequent recovery period at 37 C remaining essentially unchanged. Control bacteria kept at 3 C for 48 hours grew out quickly, without the prolonged recovery period characteristic of penicillin-damaged bacteria.

under similarly favorable conditions. (a) When pneumococci were exposed to penicillin for 2 hours, it required 2.2 hours at 37 C before they recovered sufficiently to resume multiplication (table 4). If the damaged organisms were instead placed at 3 C for 24 or even 48 hours, they failed to recover during that period, for when the organisms were then replaced at 37 C, it still required 1.9 to 2.2 hours before multiplication resumed. Control organisms not previously treated

with penicillin but stored at 3 C for a similar length of time resumed multiplication within less than one hour (cf. table 4). Similarly, (b) if the C-203 strain of *Streptococcus pyogenes* was exposed to penicillin for 45 minutes and was then placed in an unenriched beef heart infusion broth which permits only the slow multiplication of normal organisms, the number of viable organisms fell off progressively over the following 6 hours (table 5). If at the end of 2 hours defibrinated blood in adequate amounts was added to an aliquot portion, the surviving bacteria resumed multiplication only after the usual recovery period of 2 hours, indicating that there had been no recovery in the unfavorable medium. Control

TABLE 5

The failure of bacteria (C-203 strain of Streptococcus pyogenes) damaged by penicillin to recover in a relatively unfavorable medium

PREVIOUS TREATMENT OF THE BACTERIAL SUSPENSION	PERCENTAGE OF VIABLE ORGANISMS AFTER INCUBATION AT 37 C FOR							TIME (HOURS) BEFORE BACTERIA RECOVERED FROM THE TOXIC EFFECTS OF PENICILLIN AND RESUMED MULTIPLICATION
	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	
Exposed to 0.1 µg/ml for 45 min, when penicillinase was added								
(a) No further treatment.	3.2	0.7	1.8	7.0	21	176		1–2
(b) 1:50 dilution in plain beef heart infusion broth.	3.2	1.8	0.6	0.17	0.21	—	0	Progressive loss of viability without recovery
(c) 2% rabbit blood added to tube (b) after 2 hr	0.6	0.29	0.33	1.2	7.3	—	160	2±
Control suspension not previously treated with penicillin								
In plain broth...	100	115	135	—	525	—	2210	—
In blood broth.	100	215	1315		5790	—	25000	—

organisms not previously treated with penicillin, incubated in the broth medium for 2 hours, grew out immediately on the addition of blood.

DISCUSSION

The bacteriostatic effect of penicillin on staphylococci described by Bigger (1944), Parker and Marsh (1946), and Parker and Luse (1948) has here been shown to be a general phenomenon, observed with each of seven different bacterial species. Apart from variations between individual bacterial strains or species, it has been found that the rate at which the toxic effect becomes manifest is affected by the concentration of penicillin, and that the time required for the organisms to recover and resume multiplication is a function of the duration of that exposure.

The initial bacteriostatic effect of penicillin regularly preceded the death of a significant proportion of the cells, and, in general, the speed with which the penicillin exerted its bacteriostatic effect on a given strain was paralleled by the rate of its following bactericidal action. These time relationships, coupled with the fact that for a given strain the same concentrations of penicillin are necessary to produce the two effects, and the further observation that both are evidenced only at temperatures and in media conducive to growth, all suggest a close relationship between the two phenomena. Thus, penicillin may initiate chemical changes in the cell which in their first stages are reversible, and evidenced by the temporary cessation of growth. Eventually, however, the effects of the reaction chain initiated by penicillin may become irreversible, and the cell loses its viability.

A possibly significant difference in the bacteriostatic and bactericidal effects of penicillin must, however, be emphasized. The former affects all the organisms in the suspension at approximately the same rate and to approximately the same degree. (At optimal concentrations of penicillin, essentially all the organisms are affected within a relatively short time, and multiplication stops completely. Further, when the penicillin is removed the individual organisms recover more or less simultaneously, so that, when multiplication is finally resumed, within a relatively short time it is proceeding at essentially the same rate as in a control suspension of untreated bacteria.) Only occasionally does one observe a gradual resumption of multiplication in a penicillin-treated suspension. In following the bacteriostatic action of penicillin one therefore sees little evidence of significant differences between the individual bacteria of a given culture. In contrast, the bactericidal action of penicillin is characteristically an extended process. In a culture that contains, e.g., a million bacterial clumps per ml, and that begins to die under the influence of penicillin within, e.g., one-half hour, it may require as long as 48 or even 72 hours to effect total sterilization. There may thus be more than a 100-fold difference between the most susceptible and most resistant organisms, measured in terms of the time required for penicillin to render the cell nonviable.

One can only speculate as to the basis for this difference. The bacteriostatic effect may reflect a chemical reaction of penicillin with a cell component essential to continued growth and multiplication. In such a direct chemical reaction, one would not anticipate striking differences between individual bacteria. On the other hand, the widely varying rate at which the individual bacteria subsequently die may reflect the usual wide spectrum of "resistance" in the bacterial population, even if the basis of that "resistance" is as yet undefined. It is of interest in this connection that with most of the organisms studied so far, the bactericidal action proceeds at a rate consistent with a normal distribution of "resistance" (Eagle, 1949a,b), with a linear relationship between the probits of survivors and the logarithm of time.

The fact that the bacteriostatic action of penicillin regularly precedes the death of a significant number of the cells necessitates a minor revision, perhaps only semantic, in the frequently encountered statement that penicillin kills only actively multiplying cells. It was clearly indicated by Chain and Duthie (1945)

that the essential requirement for the bactericidal action of penicillin is merely that the organisms be in a medium favorable for growth and not necessarily multiplying. In the present experiments also it has been shown that penicillin begins to kill bacteria only *after* all the organisms in the suspension have stopped multiplying. A secondary reaction then takes place which results in a loss of viability gradually extending throughout the culture. It is significant that even after the bacteria have stopped multiplying under the impact of penicillin, the time required for them to recover from the toxic effects of the drug continues to increase with the duration of their exposure. The bactericidal action of penicillin may thus depend on the continuation, in a favorable medium, of certain metabolic activities in cells in which other processes that normally lead to growth and cell division have been interrupted by penicillin. The precise nature of the intracellular changes, *after* penicillin has exerted its initial bacteriostatic effect, that finally result in the death of the cell, and the nature of the slow reparative process after the removal of the drug, remain to be determined.

The therapeutic importance of the bacteriostatic effect of penicillin and of the following recovery period requires no elaboration. Soon after penicillin attains effective concentrations, the bacteria cease multiplying; and the bacteriostatic effect persists for a number of hours after penicillin has fallen to concentrations that are wholly ineffective. The duration of this postpenicillin recovery period varies from strain to strain, varies with the concentration of penicillin to which the organisms had been exposed, and varies also with the duration of that exposure. The therapeutic significance of this postpenicillin recovery period is enhanced by the fact that the recovering bacteria, damaged but not killed by the previous exposure to penicillin, are abnormally susceptible to the host defenses (Eagle, 1949b). In consequence, the bactericidal process *in vivo* continues for many hours after the drug itself has fallen to ineffective concentrations.

SUMMARY

Penicillin has been shown by previous workers to have a bacteriostatic effect on staphylococci that persists for a number of hours after the removal of the drug. As here shown, this bacteriostatic effect is not peculiar to staphylococci, but has been observed with a number of bacterial species (β-hemolytic streptococci of groups A and B, α-hemolytic streptococci, *Streptococcus faecalis*, *Diplococcus pneumoniae*, *Staphylococcus aureus*, and *Staphylococcus albus*).

The bacteriostatic effect of penicillin preceded the death of a significant proportion of the organisms, and, like the latter, was observed only under conditions favorable to the growth of the organisms. The recovery of the damaged bacteria required a similarly favorable environment.

The time required for this sublethal toxic effect to become manifest varied with the concentration of penicillin to which the organisms had been exposed and was correlated with the rate at which the bacteria were subsequently killed by the drug. With some strains rapidly killed by penicillin, its initial bacteriostatic effect was apparent within less than 5 minutes after the addition of penicillin. There was, however, no demonstrable correlation between the "sensitivity" of the

several organisms to penicillin (i.e., the concentrations necessary to exert either a bacteriostatic or bactericidal effect) and the rate at which these toxic effects were manifested.

The time required for the bacteria to recover from the toxic effects of penicillin and resume multiplication increased with the time for which they had previously been exposed to the drug. The maximum duration of this recovery period varied from 3 to 8 hours in the several species here studied.

The implications of these observations with respect to the mode of action of penicillin, and the therapeutic significance of the recovery period, are discussed in the text.

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NONSPECIFIC INHIBITION OF THE LECITHINASE ACTIVITY
OF TYPE A CLOSTRIDIUM WELCHII TOXIN

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Nagler (1939) and Seiffert (1939) independently described the production of opalescence in human serum by type A *Clostridium welchii* toxin. This reaction was found to be correlated with the lethal action of the exotoxin and inhibited by specific immune serum. MacFarlane and Knight (1941), in an analysis of the chemical reactions involved in this phenomenon, demonstrated that the α fraction was the responsible component of the toxin and that it was a lecithinase. A simple method for the titration of this lecithinase activity using filtered egg yolk lecithovitellin (LV) was developed by van Heyningen (1941). As a result of these and other investigations, convenient *in vitro* methods for estimating the potency of type A *C. welchii* toxins are available and the titrations of antibody responses following infection or immunization have been simplified.

It was noted by Nagler (1939), at the time of his initial observations, that, unlike human serum, the production of opalescence by *C. welchii* toxin did not occur with most animal serum. Several explanations of this nonreactivity of normal animal serum are possible: (1) lack of substrate in available form, (2) lack of necessary coenzymes, (3) presence of specific immune bodies, and (4) presence of naturally occurring nonspecific inhibitory substances. The experimental evidence presented here appears to support the last of these explanations.

MATERIALS AND METHODS

Toxin was prepared from a lyophilized stock culture of a type A *C. welchii* (SR-12) which was obtained from the Microbiological Institute of the National Institutes of Health. The organism was cultured in a veal infusion tryptose phosphate broth with 1 per cent added glucose. After 18 hours' incubation at 37 C in a Brewer anaerobic jar, the broth was filtered through a no. 6 Seitz pad. A single pool of the crude SR-12 filtrate, stored at 4 C in sealed ampoules, was used for all experiments.

The LV substrate was prepared by a modification of the method of van Heyningen (1941). One hen's egg yolk was mixed in 250 ml of physiological saline solution, heated at 56 C for 30 minutes, and filtered after cooling through a no. 14 Seitz pad. The resulting clear and sterile solution was kept at 4 C and was not used after 1 week of storage.

Previous methods that have been devised for the quantitative determination of lecithinase activity are dependent on a short reaction period using high concentrations of enzyme (MacFarlane and Knight, 1941; van Heyningen, 1941;

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Zamecnik *et al.*, 1947). These procedures were not considered to be readily adaptable to a study of minimal inhibitory effects. A simple serological method was used which eliminated the variable of contact time of enzyme and substrate and was sensitive for small quantities of enzyme. To test tubes containing twofold serial dilutions of the SR-12 filtrate in saline, an equal volume of LV was added. The test was read after incubation at 37 C for 18 hours and a positive reaction identified by opalescence or supernatant fat layer, the end point being taken as the last positive tube. No attempt was made to determine the degree of hydrolysis at each dilution, the result being read as positive or negative with respect to the LV saline control. The reaction was complete in all cases at the end of 18 hours and the final end point was invariably clear-cut. The lecithinase activity was found to be independent of the concentration of LV when an excess of this substrate was used.

All dilutions were made in unbuffered physiological salt solutions. Although the hydrolysis rate varies with pH and calcium ion concentration (MacFarlane and Knight, 1941), these factors were found to play a negligible role in these experiments in which the reaction times were prolonged. The pH was tested frequently and found to vary little from 6.5 and necessary trace amounts of calcium ion were present in the reacting materials.

Unless otherwise indicated, the serums used in these experiments were unheated, pooled specimens obtained by cardiac puncture in animals and venepuncture in humans. Immune rabbit serum (IRS) was produced in adult, male rabbits, by 3 weekly intravenous injections of SR-12 filtrate, the animals being bled 7 days after the last inoculation. The saline tissue extracts were prepared from freshly sacrificed normal animals. After mincing and washing, the tissues were ground as 20 per cent physiological saline suspensions in a Waring blender, and Seitz-filtered. The clear filtrates were used as test material.

EXPERIMENTAL RESULTS

The crude SR-12 filtrate produced hydrolysis of the LV solution in a dilution of 1:256. This end point was reproducible on repeated determinations. This concentration of filtrate represented approximately 0.01 MLD for 21 day-old Swiss mice.

Inhibition of LV hydrolysis. Five-tenths ml of a 1:128 dilution of SR-12 filtrate constantly produced opalescence of an equal volume of LV. At this concentration, which is designated as the twice lecithin-hydrolyzing dose (2 LHD), the toxin could be stored at 4 C in a stoppered flask for several weeks without loss of activity. Titers of inhibition of test materials were determined for this quantity of enzyme. Serial twofold dilutions of 0.5 ml of serum were made in test tubes containing 2 LHD of SR-12 filtrate. To compensate for the dilution factor of the serum, the first tube contained 0.5 ml of a 4-LHD concentration of the filtrate. To each final 0.5-ml volume, containing 2 LHD of SR-12 filtrate and falling concentrations of inhibitor, 0.5 ml of LV were added. The reactions were read after 18 hours of incubation at 37 C and the titers of inhibition recorded as the last tube that showed clear LV solution. Four controls were used for each series of experiments: (1) *positive control*—2 LHD and LV, (2) *negative*

control—test material and LV, (3) *serum control*—serum or tissue extract and 2 LHD, and (4) *saline control*—saline and LV.

Normal serums of rabbits, mice, and guinea pigs and normal human serum (NHS) were assayed for lecithinase inhibition by this method. For comparison, titrations of inhibition of IRS and saline tissue extracts of normal animals were done. Normal rabbit serum (NRS) exhibited a pronounced inhibitory effect on the 2-LHD hydrolysis of LV; the serums of normal guinea pigs and mice were somewhat less effective. The reaction was unaffected by NHS, which became opalescent when incubated alone with this quantity of enzyme; all normal animal serum controls were negative. Saline extracts of tissues of normal rabbits and mice produced an inconsistent inhibition of a lower order than the serums of these species. The serum of immunized rabbits exhibited the expected high titer of inhibition (table 1).

Inhibition of hemolytic activity. Confirmatory evidence of this inhibitory phenomenon was sought by the use of a different substrate-indicator system. The correlation between toxicity and lechithinase activity of type A *C. welchii* filtrates

TABLE 1

Effect of serum and saline tissue extracts on the lecithinase hydrolysis of LV

(Results expressed as the reciprocal of the titer of inhibition of 2-LHD SR-12 filtrate)

SPECIES	INHIBITOR					
	Normal serum	Immune serum	Brain	Lung	Muscle	Liver
Human	0					
Mouse.	8		0	2	0	0
Guinea pig.	8					
Rabbit.	64	1,024	2	0	8	16

extends to their capacity to hemolyze red cells (MacFarlane, Oakley, and Anderson, 1941). However, at least two distinct fractions of the crude toxin are known to possess hemolytic activity, the α-fraction or lecithinase and a "purely hemolytic" θ-toxin (Oakley and Warrack, 1941). It has been postulated that α-toxin causes lysis of erythrocytes by hydrolysis of the lecithin of the cell membrane (MacFarlane, Oakley, and Anderson, 1941).

The minimal hemolytic dose of SR-12 filtrate for sheep cells was determined employing a 2 per cent suspension of erythrocytes in physiological saline solution containing 0.005 M $CaCl_2$. Twofold dilutions of materials being tested for inhibitory activity were made in twice the concentration of SR-12 filtrate necessary to produce a 2+ hemolysis, incubated at 37 C, and the titers read as the last tube to show inhibition of hemolysis of an equal volume of the red cell suspension.

Table 2 demonstrates that the serum titers were greater at 4 hours than at 18 hours, indicating that some of the inhibitory capacity was lost in the longer period. In general, however, the results are similar to those obtained with LV hydrolysis inhibition. The IRS was far more active in preventing hemolysis than was the NRS. Removal of ionized calcium by the addition of sodium citrate

completely inhibited the hemolytic activity of the toxin, but this effect did not diminish in 18 hours. Inhibition of hemolysis is also exhibited by LV solution. At 4 hours this effect is marked, but with enzyme hydrolysis of LV, its inhibitory activity is destroyed. This is presumed to be due to competition of the two substrates for the enzyme during the early part of the reaction.

Effect of heat on serum inhibitors. If the presence of inhibitors is the sole factor concerned in the nonreactivity of normal animal serum, their destruction should allow the reaction to proceed without the addition of LV. Heating NRS to 65 C for 2 hours prior to the addition of an equal volume of the undiluted SR-12 filtrate, and then incubating for 40 hours at 37 C, produced the reaction of opalescence. Even with this preliminary heating, it is evident that greater enzyme

TABLE 2

Inhibitory effect of various materials on the minimal sheep erythrocyte hemolytic dose of SR-12 filtrate

(Results expressed as the reciprocal of the inhibition titer of 2+ hemolysis)

TIME OF 37 C INCUBATION	INHIBITOR				
	5% Na-citrate	NRS	IRS	LV	Sheep RBC*
4 hours..................	>1,024	32	512	64	
18 hours.................	>1,024	4	128	0	2

* Concurrent inhibition of LV hydrolysis.

TABLE 3

Relative heat lability of serum inhibitors

SERUM	TEMPERATURE AND TIME OF EXPOSURE				
	4 C	37 C, 18 hours	56 C, 30 min	56 C, 100 min	100 C, 10 min
Normal rabbit.... .	64	8	16	4	0
Immune rabbit.	1,024	2,048	1,024	1,024	
Normal mouse ..	8	2	2	2	0
Normal guinea pig... .	8	0	0	0	0

concentration and a longer contact period are necessary to demonstrate the reaction than with NHS or LV.

The influence of heat on the serum inhibitors of the 2-LHD hydrolysis of LV was determined and compared with IRS. As seen in table 3, normal serum inhibitory activity is progressively diminished with increasing temperature and time of exposure. In contrast to this is the marked heat resistance of specific antienzyme. The inhibitory activity of NRS could not be restored by guinea pig complement.

The lecithinase activity of type A *C. welchii* toxin is only partly inactivated by boiling for 10 minutes or by exposure to 37 C for 18 hours. This stability to heat was completely altered by mixing the enzyme with normal serum. In the presence of subinhibitory doses of NRS, SR-12 lecithinase activity is completely destroyed at 56 C for 30 minutes. This procedure did not decrease the enzyme activity when the exposure was 37 C for 18 hours (table 4).

Effect of inhibitors on toxicity for mice. The correlation between the lecithinase activity of α-toxin and its lethality has been amply demonstrated as has the relation of the antilecithinase effect of immune serum to its toxin-neutralizing capacity (Oakley and Warrack, 1941). An attempt was made to detect gross differences in the *in vivo* protective action of some of these inhibitory materials.

Equal amounts of lyophilized SR-12 filtrate were reconstituted in saline, IRS, LV, NRS, and 10-times-concentrated NRS. These solutions were allowed to stand at room temperature for 1 hour prior to injection intravenously of 0.3-ml quantities into 21 day-old Swiss albino mice. This volume of saline-reconstituted toxin represented 5 MLD. Control NRS and LV inoculations were also made; they had no effect. Deaths were recorded at half-hourly intervals and the mean survival time was computed for each group.

As expected, IRS completely protected the mice. There was no protection afforded by LV or by either the unconcentrated or lyophilized NRS. Although the mean survival times of mice injected with dried SR-12 toxin reconstituted in LV or NRS were somewhat shorter than the saline-reconstituted toxin controls, these results were within the limits of the experimental error.

TABLE 4

Loss of heat resistance of SR-12 lecithinase when mixed with equal amount of NRS

	TEMPERATURE AND TIME OF EXPOSURE				
	4 C	37 C, 18 hr	56 C, 30 min	56 C, 100 min	100 C, 10 min
SR-12 + saline	512	64	128	64	64
SR-12 + NRS	512	256	0	0	0

DISCUSSION

With preliminary heating and prolonged incubation with SR-12 filtrate, NRS will exhibit the reaction of opalescence. This serves to indicate that its apparent nonreactivity is not due to the absence of substrate or other materials essential for this enzymatic phenomenon. The most likely explanation of this lecithinase-blocking action of normal animal serums would seem to be the presence of "nonspecific" inhibitors. These inhibitors are distinguished from specific antibody on the basis of differential heat stability.

The mechanism of action of immune serum in preventing the lecithinase hydrolysis of lecithin is presumably through the formation of an enzyme-antibody complex. Zamecnik and Lipmann (1947) have also shown that *C. welchii* lecithinase will form a complex with lecithin to the exclusion of subsequently added antibody. This formation of an enzyme-substrate complex appears to be confirmed by the studies presented here, since LV will inhibit the action of the enzyme on another substrate, sheep erythrocytes. However, the lecithinase-LV complex is dissociable with hydrolysis of LV, whereas the enzyme-antibody complex is stable.

The inhibitor of normal serum appears to act directly on the enzyme and not the substrate, the LV concentration being far in excess of either lecithinase or

inhibitor. This inhibitory action may be due to the formation of an enzyme-inhibitor complex. If this hypothesis is correct, this union differs from that of lecithinase and lecithin in its relative nondissociability in comparable incubation periods. Also, no action of the enzyme on the inhibitor is demonstrable; the loss of inhibitory activity of NRS when incubated at 37 C with SR-12 filtrate is no more than can be accounted for by its lability at this temperature.

The loss of heat resistance of lecithinase in the presence of subinhibitory doses of NRS may provide additional evidence of complex formation. It is possible to explain this alteration of stability as either an increase in the extent of the union at higher temperatures or as the result of greater lability of the complex.

Of the species studied, there is no correlation between the level of titratable serum inhibitor and susceptibility to the toxic effects of *C. welchii* filtrates. It is also evident that under the experimental conditions imposed, no neutralization of the lethal action of the toxin for mice is demonstrable by NRS or LV.

SUMMARY

The serums of normal rabbits, guinea pigs, and mice produce a slight but definite inhibition of the lecithinase activity of type A *Clostridium welchii* toxin. The reaction is not affected by normal human serum.

Both normal rabbit serum and lecithovitellin inhibit the hemolytic action of the toxin; the effect of normal rabbit serum is more prolonged.

The nonspecific serum inhibitor is distinguished from antibody by its differential heat lability. In the presence of subinhibitory doses of normal rabbit serum, the marked resistance to heat of the lecithinase is lost. The possible relation of these effects to the formation of an enzyme-inhibitor complex is discussed.

Neither normal rabbit serum nor lecithovitellin diminishes the lethal action of the crude toxin.

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THE RELATIONSHIP OF SHIGELLA DISPAR TO CERTAIN COLIFORM BACTERIA

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This report deals with serological and biochemical relationships between members of the genus *Escherichia* and *Escherichia*-like paracolon bacteria and the microorganisms that are usually classified as *Shigella dispar*. These studies are part of an investigation on the interrelationships within the family *Enterobacteriaceae* that was started in 1942 and which is still in progress.

Shigella ceylonensis A and *Shigella ceylonensis* B were isolated by Castellani in 1904 and 1905. In 1912 this investigator described another bacterium that was later named *Shigella madampensis*. *S. ceylonensis* A was subsequently found to be the same as the microorganism described by Duval (1904), Sonne (1915), and d'Herelle (1916). This species is now known as *Shigella sonnei*. (For references to these earlier publications, see Soule and Heyman, 1933). Andrewes (1918) studied a group of *Shigella* and *Shigella*-like microorganisms and proposed a new species, "*B. dispar*," in which were placed all the lactose-positive shigellae "... without prejudice as to the number of types or species which may thus be included." Levine (1920) reported that the indole-negative "dispars" of Andrewes were culturally the same as the Sonne-Duval bacillus (*Shigella sonnei*).

The work of Carpenter and Stuart (1946) and of Carpenter (1943) did much toward ending the confusion extant in the literature pertaining to the indole-positive, lactose-positive shigellae. Their investigations showed that *S. madampensis* and *S. ceylonensis* B were composed of a group of closely related microorganisms. Carpenter and Stuart used the name *S. dispar* for this group of bacteria and included *S. dispar* I (*S. madampensis*) and *S. dispar* II (*S. ceylonensis* B) in it. They reported three serotypes of *S. dispar* II (IIa, IIb, and IIc) and Carpenter (personal communication, 1946) added a fourth, *S. dispar* IId. The various types of *S. dispar* were kindly supplied by Dr. Carpenter and the results reported here confirm the relationships enumerated by Carpenter and Stuart (1946) in all important aspects.

METHODS AND RESULTS

Since 1943 a relatively large number of cultures of *S. dispar* and related microorganisms have been isolated and studied. Among the cultures were found typical coliform and paracolon bacteria that were related to, and in some cases serologically identical with, *S. dispar* serotypes. The cultures reported here are representative of that group.

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The methods used were those described earlier (Ewing, 1946). All of the microorganisms referred to as aerogenic cultures produced acid and gas from glucose, mannitol, and rhamnose. Dulcitol and sorbitol were utilized by most cultures. Lactose was fermented with gas production by all cultures except 983, 984, and 897, although the fermentation was usually delayed. Slow utilization of sucrose by cultures 818, 859, and 1478 was observed. All cultures except 983 formed indole. Adonitol was attacked only by culture 984, and cultures 818 and 983 grew on Simmons' citrate agar. Salicin was not fermented. Motility was demonstrated only in culture 983. The biochemical reactions outlined above are characteristic of cultures 718, 721, 818, 859, 866, 887, 897, 923, 953, 983, 984, and 1478, the serological properties of which are given in table 1. These microorganisms are selected representatives of a group of similar coliform bacteria which were studied.

The antisera employed were derived from all of the S. dispar serotypes and several of the aerogenic bacteria. When two or more cultures were tested in an antiserum, master dilutions were employed. All agglutinative tests were incubated at 52 to 53 C for 16 to 18 hours and were read with the unaided eye. The presence of alpha antigens and agglutinins was eliminated by appropriate tests.

Preliminary tests indicated that culture 897 had little or no effect upon the agglutinative titer of antiserum 721. Culture 818 removed all agglutinins for itself and lowered the titer from 1:40,960 to 1:2,560 for the antiserum culture 721. Adsorption of this antiserum by cultures 718, 721, 859, 866, 953, and 1478 resulted in the removal of all agglutinins. This indicated that these microorganisms possessed major antigenic relationships to culture 721. The possibility that these bacteria might contain antigens in addition to those of culture 721 was not eliminated, however.

The serological reactions of S. dispar serotypes and of representative aerogenic coliform and paracolon cultures are given in table 1. The results with S. dispar cultures are essentially the same as those reported by Carpenter and Stuart (1946). It was apparent from tests in unadsorbed antisera that more than one serological type existed among the aerogenic cultures. Therefore, a systematic study was made to determine the antigenic structure of these cultures and to identify the factors common to S. dispar serotypes.

Reciprocal adsorptive tests were made with antisera prepared with type S. dispar cultures and against the aerogenic bacteria. The results of the more important of these tests are given in table 1.

Examination of these data reveals that aerogenic culture 721 shares major antigenic factors with S. dispar I (S. 171) but is not identical with it. The converse of this is also true, that is, a specific fraction is left in antiserum 721 following adsorption with S. dispar I. Culture 818 is serologically identical with S. dispar I (S. 171) as demonstrated by reciprocal adsorptive tests. Aerogenic cultures 718 and 866 differ from 721 in that they contain antigens in common with S. dispar I that are not shared by culture 721.

Culture 897 is serologically identical with culture S. 315, designated S. dispar IId by Carpenter (1946). Serologically these microorganisms are the same, as demonstrated by reciprocal adsorptive tests, as culture 609 and other cultures

TABLE 1

Agglutinative titers of Shigella dispar serotypes and representative acrogenic cultures

ANTISERUM	ADSORBED BY	TEST SUSPENSIONS									
		Shigella dispar					Representative aerogenic cultures				
		S. 171	S. 167	S. 205	S. 231	S. 315	718	721	818	866	897
S.171		40,960*	2,560	1,280	2,560	1,280	40,960	40,960	40,960	40,960	1,280
(I)	721	2,560	0	0	0	0	640	0	1,280	320	0
	818	0	0	0	0	0	0	0	0	0	0
	866	2,560	160	160	0	0	0	0	1,280	0	0
	897	40,960	320	320	320	0	40,960	40,960	40,960	40,960	0
S.167		2,560	40,960	20,480	40,960	2,560	5,120	2,560	2,560	0	5,120
(IIa)	721	160	20,480	20,480	20,480	5,120	0	0	160	0	2,560
	818	0	20,480	10,240	10,240	640	0	0	0	0	640
	866	160	40,960	40,960	40,960	5,120	0	0	160	0	2,560
	897	160	40,960	20,480	40,960	0	160	160	160	320	0
S.205		80	10,240	20,480	10,240	1,280	320	80	160	0	1,280
(IIb)	721	0	20,480	40,960	40,960	2,560	0	0	0	0	2,560
	818	0	10,240	10,240	5,120	320	0	0	0	0	640
	866	0	40,960	20,480	40,960	2,560	0	0	0	0	2,560
	897	80	20,480	20,480	20,480	0	80	160	80	80	0
S.231		1,280	40,960	40,960	40,960	5,120	10,240	2,560	5,120	10,240	10,240
(IIc)	721	0	40,960	40,960	40,960	5,120	0	0	0	0	10,240
	818	0	40,960	40,960	40,960	10,240	0	0	0	0	5,120
	866	0	40,960	40,960	40,960	5,120	0	0	0	0	2,560
	897	2,560	40,960	40,960	40,960	0	5,120	2,560	2,560	5,120	0
S.315		320	10,240	10,240	10,240	40,960	640	160	160	160	40,960
(IId)	721	0	20,480	40,960	20,480	40,960	160	0	0	0	40,960
	818	160	10,240	10,240	10,240	20,480	160	0	0	160	40,960
	866	0	5,120	5,120	5,120	2,560	0	0	0	0	40,960
	897	0	0	0	0	0	0	0	0	0	0
721		40,960	640	640	640	5,120	10,240	40,960	20,480	40,960	10,240
	S.171	0	0	0	0	0	640	2,560	0	640	0
	S.167	20,480	0	0	0	0	10,240	20,480	20,480	20,480	160
	S.205	10,240	0	0	0	0	10,240	20,480	20,480	20,480	160
	S.231	10,240	0	0	0	0	10,240	10,240	5,120	10,240	160
	S.315	2,560	0	0	0	0	5,120	2,560	2,560	2,560	0
721	818	0	160	0	0	160	2,560	2,560	0	1,280	0
	866	0	0	0	0	0	0	0	0	0	0
	897	20,480	0	0	0	0	20,480	20,480	10,240	20,480	0

0, no reaction at 1:40 dilution.

* Agglutinative titers are expressed as the reciprocal of the highest dilution in which agglutination occurred.

of the paracolon type sometimes designated *Shigella alkalescens* II (DeAssis, 1947). The relationships of the latter type of bacteria to shigellae will be dis-

cussed elsewhere. It suffices to state that the so-called *S. alkalescens* II is not a *Shigella alkalescens* type, nor, in fact, any *Shigella* type, but is a member of the *Escherichia* group (Ewing, unpublished data; Kauffmann and Frantzen, personal communication).

The results of the reciprocal agglutinin-adsorption tests with all the antisera prepared with aerogenic microorganisms are not included in table 1. In general, the results of these tests mirror those of the table. For example, culture S. 171 (*S. dispar* I) removes all agglutinins from 818 antiserum and *S. dispar* IId (S. 315) adsorbs all antibody from antiserum 897.

DISCUSSION

Comparison of the biochemical reactions of typical *S. dispar* cultures and the aerogenic coliform bacteria reveals that the only essential difference between them is gas production. The results of the serological tests outlined above show clearly that the relationships go beyond biochemical similarity. Some of the coliform microorganisms are identical with certain *S. dispar* types and others are antigenically closely related. Thus, *S. dispar* cultures might be regarded as anaerogenic coliform or paracolon bacteria.

It is possible to identify the factors involved in these relationships and to write an antigenic formula for *S. dispar* cultures and the related coliform bacteria. Formulas are not included at this time because it is advisable first to identify these antigens with those of the *Escherichia* O antigen groups of Kauffmann (1947). It is clear that the antigenic factors we have identified are separate O antigens related to certain of the *Escherichia* O groups (Kauffmann and Frantzen, personal communications). Further studies on the relationships of *S. dispar* serotypes and the aerogenic cultures mentioned above to the delineated O antigen groups of Kauffmann are in progress.

SUMMARY

The biochemical and serological relationships between *Shigella dispar* serotypes and certain coliform and paracolon microorganisms are reported.

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FIBRINOLYSIS IN GRAM-NEGATIVE BACILLI

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The bacteriology of peritonitis complicating appendicitis has been carefully studied with varying emphasis on the role of each of the more commonly found organisms. Steinberg (1944) and others (Rademaker, 1933; Altemeier, 1938) have emphasized the role of the gram-negative bacilli in peritonitis. Bower and his associates (1928, 1938) have shown the importance of the clostridia, particularly *Clostridium welchii*, both experimentally and clinically, whereas Meleney and his associates (1931, 1932) have re-emphasized the importance of gram-negative bacilli and have shown the importance of synergism of two or more organisms in the mortality associated with peritonitis.

Recently interest has been aroused in the role of fibrinolysis in experimental appendiceal peritonitis by the observations of Kay and Lockwood (1947), who have found little correlation between mortality and the initial flora in the appendix but have emphasized rather the prognostic importance of the fibrinolytic and antifibrinolytic activity of the plasma. Increased fibrinolytic activity before onset and during the disease was of unfavorable prognosis.

Christensen (1945), continuing the original work of Milstone (1941), has demonstrated that fibrinolysis by *beta*-hemolytic streptococci was caused by the activation of plasma fibrinolysin by a specific factor in culture filtrates which he has named *streptokinase*. A similar factor apparently is produced by certain strains of clostridia (Lockwood, 1946; Reed, Orr, and Brown, 1943). Fibrinolysis has also been reported by Neter and Witebsky (1936) to occur with cultures of coliforms, *Pseudomonas pyocyaneus*, *Proteus*, and *Streptococcus enteritidis*. An analysis of the fibrinolytic capacities of various strains of gram-negative bacilli isolated in this laboratory was undertaken to supplement other data on these organisms.

METHODS AND MATERIALS

(1) The main methods used depend upon observing the disappearance of a fibrin clot. The first method employed was described by Tillett and Garner (1933) in their original report on the fibrinolytic activity of streptococci. In some cases the plasma was clotted first, and the culture was layered on top of the clot. The tubes were incubated at 37 C for about 24 hours in most cases. The degree of liquefaction was recorded as 1 to 4 plus, 1 plus being given for each one-fourth of the length of the clot that was lysed in the tube. The decrease in viscosity of gelatin solutions that resulted from the enzyme activity was determined with Ostwald viscosimeters at 37 C in the semiquantitative experiments.

(2) The bacteria employed were organisms obtained from surgical patients

and dogs with peritonitis, and a strain of hemolytic streptococcus, group C, strain P-168, which was used as a source of streptokinase. This is a stock strain from the Department of Bacteriology and was obtained through the courtesy of Dr. H. E. Morton.

(3) The media employed were brain-heart infusion broth (Difco), nutrient broth, and gelatin (Difco). In some mixtures 2 per cent glucose was added to these media, but in others it was omitted.

(4) The substrates used to test lytic activity were 1:4 or 1:5 diluted, citrated dog plasma clotted with human thrombin (Sharp and Dohme). Gelatin (Knox P-20) was used to follow the decrease in viscosity with lysis.

(5) Streptokinase was prepared by a modification of the method of Christensen (1945). Extractions of cultures of *Escherichia coli* were prepared in similar fashion, and by freezing and thawing or prolonged grinding, in order to break up the cells.

(6) The plasminogen was prepared by one-third saturation of human serum with ammonium sulfate. The precipitate was washed in one-third saturated ammonium sulfate, and dissolved in one-tenth the original volume of physiological saline.

It is of interest to note that the plasminogen used in these determinations was prepared from lyophilized serum which had been stored at 5 C since 1939. Active plasminogen preparations could be obtained by ammonium sulfate precipitation, but active plasmin was not found when chloroform extraction was used as it is in fresh serum. Christensen (1946) reported that plasminogen in fresh plasma is destroyed by chloroform. The destruction of the enzyme by chloroform is important in view of the fact that chloroform has been employed to activate plasminogen, and Kay and Lockwood (1947) have recently used chloroform extraction as a test for total proteolytic activity in the blood.

(7) Phosphate buffer (M/10) at pH 7.4 with added 0.9 per cent sodium chloride was used as the solvent for the extraction of the bacterial cultures and for redissolving the plasminogen. Lactate buffers at M/5 concentration and various pH values were also used in some of the experiments.

(8) The indicators used were phenol red, bromphenol blue, nitrazene paper, and "Harleco" liquid indicator.

(9) Bacterial counts were made by the method of Wadsworth (1927), and turbidimetrically.

(10) Inhibitors were added to the cultures to be tested immediately before layering.

(11) Cell-free filtrates were obtained by Seitz filtration.

EXPERIMENTAL RESULTS

Sixty strains of gram-negative bacilli were tested for fibrinolytic capacities, and virulence for mice was determined by intraperitoneal injection for 50 of them. The over-all results are shown in table 1. Fibrinolytic and hemolytic capacities of *Escherichia coli* seem to be associated with virulence. Of these two, fibrinolytic capacity seemed to be the more important because virulence and

fibrinolytic activity disappeared simultaneously in artificial media even though hemolytic capacity remained, and of the ten most virulent strains eight showed no hemolytic capacity whatsoever. Virulence in the non-lactose-fermenting group seemed to bear no relation to hemolytic or fibrinolytic capacities.

It is noteworthy that the virulent strains of *Escherichia coli* compared favorably in virulence with clostridia of various types isolated from similar sources. These strains were resistant to penicillin at levels well above those commonly attained therapeutically in body fluids, and some were resistant to streptomycin.

TABLE 1

Correlation of virulence with hemolytic and fibrinolytic capacities

STRAIN	ORIGIN	VIRU-LENCE IN MILLIONS	HEMOL-YSIS	FIBRI-NOLYSIS	PENICILLIN SENSITIVITY	STREP-TOMYCIN SENSI-TIVITY	TYPE
18 strains killing mice at 10 or less			38%+	77%+			
Goldstein........ ...	Man	10	0	0	50	2	Nonlactose
346	Man	5	0	0	50	100	*Salmonella*
DL₂. .	Dog	5	0	0	Resistant	—	Nonlactose
DE	Dog	10	+	0	1,000	—	Nonlactose
C₂.	Dog	2	0	+	100	4	*A. aerogenes*
Steinberg. ...	300	2	+	+	50	3	*E. coli*
M₄	Dog	2	0	+	50	6	*E. coli*
E₁ . ..	Dog	4	0	+	20	4	*E. coli*
K₁ . .	Dog	4	0	+	50	2	*E. coli*
DK_T	Dog	5	0	+	100	—	*E. coli*
Osborne .. .	Man	6 5	0	+	1,000	3	*E. coli*
B₄..... .	Dog	6 5	0	+	500	3	*E. coli*
C₇.......	Dog	7	0	+	100	—	*E. coli*
P₃ .. .	Man	10	+	+	20	2	*E. coli*
288 . .	Dog	10	+	+	50	100	*E. coli*
DL₁ . .	Dog	10	+	0	Resistant	—	*E. coli*
DG₂ .	Dog	10	0	0	300	—	*E. coli*
N₂ .	Dog	10	0	0	100	3	*E. coli*
32 strains requiring more than 10			72%+	32%+			

It was observed many times during the course of the study that clotting would not occur if cultures at low pH were added to the plasma. However, when these cultures were neutralized before mixing, clotting occurred normally. This, coupled with the observations of others (Dennis and Adham, 1937; Tillett, 1937), was considered as evidence that this anticoagulant effect was related to low pH and was entirely separate from fibrinolysis.

Lysis was usually associated with a fall in pH early in the period of incubation, which suggested that the whole process might be accounted for by the acidity resulting from bacterial growth (figure 1). This was determined not to be the case by layering sterile lactate buffer at descending pH over plasma clots and determining the pH at which lysis occurred (table 2).

Lysis proceeded at equal rates with the acid neutralized and unneutralized

TABLE 2
Lysis of clots by lactate buffer

	pH								
	4.8	4.5	4.2	4.0	3.9	3.7	3.5	3.3	2.9
8 hours....	0	0	0	1+	1+	2+	2+	2+	2+
24 hours...........	0	0	±	2+	2+	3+	2+	2+	2+
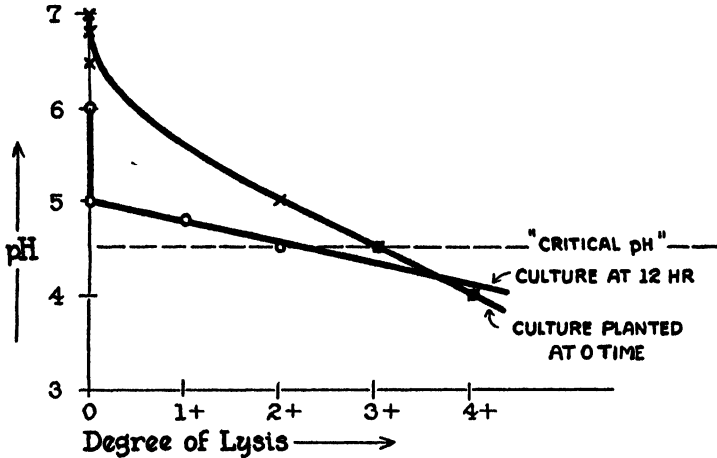
Figure 1. The relation of fibrinolysis to pH in culture of *E. coli* 428. Two and five-tenths ml of a 12-hour culture of *Escherichia coli* 428 in brain heart infusion broth with 2 per cent glucose and 2.5 ml of recently inoculated brain heart infusion broth with 2 per cent glucose were layered over a 2.5-ml clot in separate sterile test tubes. pH determinations, using nitrazene paper and "Harleco" indicator, were made at intervals.
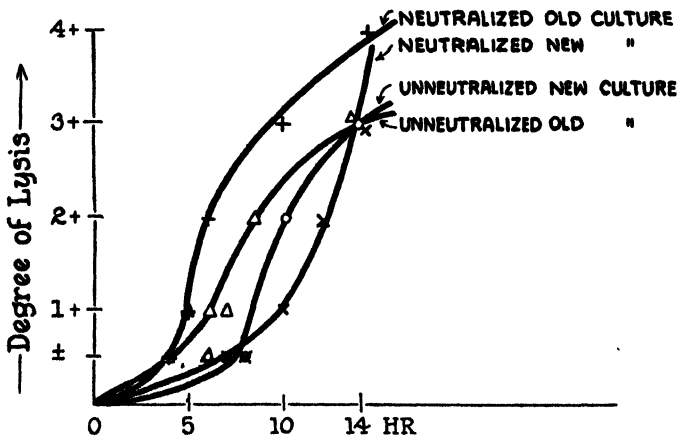
Figure 2. The effect of neutralizing acid in the culture on fibrinolysis by *E. coli* 428. Two tubes were set up with a 2.5-ml clot, over which was layered 2.5 ml of extract broth with 2 per cent glucose. This broth was inoculated with *Escherichia coli* 428 and incubated at 37 C. Two additional tubes were set up as above but with 2.5 ml of a 42-hour culture of *Escherichia coli* 428, which had been stored at 5 C, layered over the clot. The acid was neutralized with 2 N sodium hydroxide as it formed in one tube, but in the other tube it was allowed to increase without neutralization.

(figure 2). Ancillary evidence against acid's being the only cause of lysis is found in the frequent observation of loss of fibrinolytic capacity of *Escherichia coli* maintained on artificial media with retention of acid-forming properties.

Cell-free filtrates of cultures of coliforms were found to have no fibrinolytic activity both with intact bacilli and after repeated rapid freezing and thawing of the cultures in an attempt to break down the cells. The possibility that *Escherichia coli* secreted an activator of the character found by Christensen (1945) to be formed by hemolytic streptococci was considered. To test this hypothesis a vigorously fibrinolytic strain, *Escherichia coli* 428, was grown in 1 liter of brain heart infusion broth with 2 per cent glucose. Acid was neutralized with 5 N sodium hydroxide as it formed. After growth stopped, the supernatant fluid
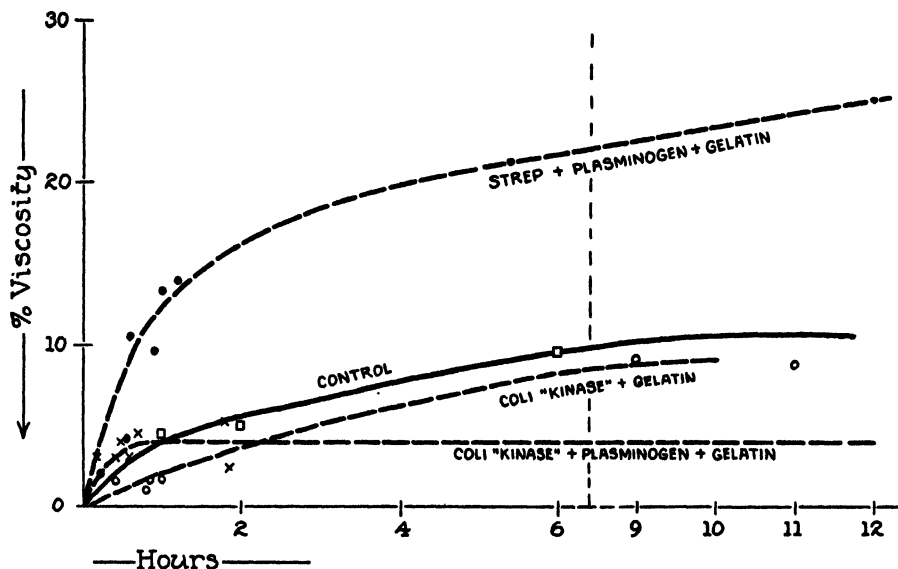
Figure 3. The fibrinolytic activity of extracts of culture of *E. Coli* 428. The fibrinolytic activity of cell-free extracts of *Escherichia coli* 428 with and without added plasminogen are compared with the effects of streptokinase in a similar system.

was decanted from the bacterial sediment and saturated with ammonium sulfate. The precipitate was collected and dissolved in phosphate buffer at pH 7.4. One-half ml of this preparation was mixed with 1.0 ml of plasma, and, after clotting, observed for fibrinolysis. None was found to occur.

The production of proteolytic enzyme by *E. coli* 428 was considered as a possible explanation of this phenomenon. Therefore gelatin media (Difco) with a solution of 2 per cent glucose was inoculated with this organism. No lysis of the gelatin occurred over a period of 3 weeks, although good growth was obtained.

Semiquantitive tests were undertaken to determine if any significant proteolysis occurred with the protein extract of the cultures. Decrease in viscosity of gelatin solutions was measured. To test for fibrinolytic activity of the type found by Christensen (1945) these experiments were repeated with the addition

TABLE 3

Fibrinolysis with various inhibitors

SUBSTANCE	COAGULATION			FIBRINOLYSIS		
	In vitro	*In vivo*	Alone	ChCl₃ act.	Strep.	Coli
Polysulfonic acids						
Heparin	−	−	0	−	−	0
Chlorazole	−	−	0	−	−	−
Trypan blue	−	−	0	−	−	+
Polyamino heterocyclic bases						
Methylene blue	+	0	0	−	−	−
Toluidine blue	+	0	0	−	−	−
9-Aminoacridine	+	0	0	−	−	−
Lipids						
Unsat. fatty acids	0	0	0	−	Not run	Not run
Cholesterol	0	0	0	−	−	−
Cholesterol esters	0	0	0	0	0	−
Mixed phospholipids	0	0	0	0	0	Not run
Cephalin	0	+	0	0	0	Not run
Thromboplastin	+	+	0	−	−	−
Lyso phosphatides	0	0	0	0	−	+
Compounds active in clot formation						
Vitamin K (2-methyl-1,4-naphthoquinone)	0	+	0	Not run	0	Not run
Thrombin	+	+	0	−	−	−
Dicoumarin	0	−	0	Not run	0	Not run
SH active						
Adenylic acid	−	Not run	0	+	+	0
KCN	−	Not run	0	+	+	+
Cystine	0	0	0	−	−	−
Cysteine	−	Not run	0	0	+	0
Antibiotics						
Sulfonamides						
Sulfanilamide	0	0	0	0	+	+
Sulfasuccidine	0	0	0	0	+	+
Sulfapyridine	0	0	0	Not run	0	−
Sulfathiazole	0	0	0	Not run	0	−
Sulfadiazine	0	0	0	Not run	+	+
Others						
Penicillin G	Not run	+	0	−	−	−
Streptomycin	+	Not run	0	−	−	−
9-Aminoacridine	+	0	0	−	−	−

of plasminogen derived from human plasma. The data obtained are shown in figure 3. There was no lysis of the gelatin comparable to that found with streptokinase under the conditions of the experiment.

Since whole cultures were fibrinolytic and since the ammonium sulfate precipitate was not, it seemed that there might be an active, nondialyzable substance in the supernatant. Accordingly the supernatant fluid was dialyzed against water until it was free of ammonium sulfate. The final solution showed no fibrinolytic activity when tested by the usual methods.

DISCUSSION

Too little attention has been paid in the past to the fibrinolytic activity of gram-negative organisms. Anticoagulant and fibrinolytic activities have been confused by many previous investigators. Neter and Witebsky (1936) reported fibrinolytic activity for cultures of many organisms, both gram-positive and gram-negative, including *Escherichia coli*, if 2 per cent glucose were included in the medium. These investigators apparently considered the test for fibrinolysis as positive if failure of coagulation occurred on the addition of calcium to mixtures of plasma and cultures. In a later study Witebsky and Neter (1936) reported two fibrinolysins for streptococci, one active in clot dissolution, the other in preventing clot formation. This clot inhibition had been previously reported for streptococci by other investigators (Dennis and Berberian, 1934; Tunnicliff, 1936). Dennis and Adham (1937) further studied the anticoagulant effect of streptococci grown in glucose broth and concluded that the inhibition of clotting was primarily due to lactic acid, and the determining factor was total acid rather than low pH. Tillett (1937) studied the anticoagulant and fibrinolytic activity of *Streptococcus hemolyticus*, *Streptococcus viridans*, and pneumococci. He concluded that the anticoagulant effect was due to pH below 5.0. Our results confirm Tillett's point of view.

Fibrinolytic activity was found in 31 of 60 strains of *Escherichia coli* tested by our methods. The experiments herein reported show that fibrinolysis does occur with *Escherichia coli*. There are several differences between *Escherichia coli* fibrinolysis and hemolytic streptococcus fibrinolysis, however. The lysis by *Escherichia coli* proceeds at a slower rate, and instead of starting as a generalized softening of the clot, followed by the disappearance of formed fibrin, it begins on the surface of the clot and proceeds from there. Lysis with streptococci occurs when they are grown on 0.05 per cent glucose media, but *Escherichia coli* will not consistently produce fibrinolytic activity at this glucose level. The extracts of cultures of *Escherichia coli* were inactive, but the cultures of the hemolytic streptococcus always yielded extracts that were active. No lysis occurred with *Escherichia coli* cultures in the absence of living, metabolizing bacteria, which may indicate that the process is related to the intrinsic enzyme systems of the bacteria. There was no effect on gelatin by any of the gram-negative bacilli studied. In table 3 it may be seen that various substances inhibited lysis by streptococci but increased lysis by *Escherichia coli*. Other substances, for example, cysteine and adenylic acid, showed the opposite effect. These differences support the contention of Tillett (1938) that no fibrinolytic substance equivalent to that of the hemolytic streptococci is found in *Escherichia coli*.

There is some positive correlation between fibrinolysis and virulence. However, the problem of *in vivo* activity is not solved in these experiments. In order to

produce maximum and consistent lysis with *Escherichia coli*, glucose is required in the medium in concentrations far exceeding the 100 mg per cent found in the body; and if the organisms metabolize *in vivo* by the same mechanisms used *in vitro*, it is extremely unlikely that much lysis of fibrin can occur. It may be that the *in vitro* fibrinolysis simply reflects some difference in the organism giving it greater pathogenic powers.

SUMMARY

Dissolution of fibrin by strains of *Escherichia coli* occurs.

This fibrinolytic capacity is separate and distinct from the acid-forming properties of the strains.

The fibrinolytic property is more often seen in strains most virulent for mice.

The lysis proceeds without significant trypsinlike activity.

The process differs from that seen with streptococci in several other important respects: (1) The lysis with coliforms is slower. (2) No cell-free extract was found to have activity. (3) To produce lysis the coliforms are best grown on a high glucose medium. (4) Significant differences in inhibition by trypan blue, lysophosphatides, adenylic acid, cysteine, and two sulfonamides exist.

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PHOTOREACTIVATION OF ULTRAVIOLET-IRRADIATED ESCHER-ICHIA COLI, WITH SPECIAL REFERENCE TO THE DOSE-REDUCTION PRINCIPLE AND TO ULTRAVIOLET-INDUCED MUTATION[1]

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A sufficient dose of ultraviolet light (2,537 A) inactivates most microorganisms. Exposure of inactivated cells to suitable visible light results in the recovery of a large portion of the cells from their otherwise fatal ultraviolet-induced injury. The discovery of light-induced recovery (Kelner, 1949) and its confirmation for bacteriophage by Dulbecco (1949) gives us fresh hope for solving the fundamental radiobiological problems of the lethal and mutagenic action of ultraviolet radiation. The effect of reactivating light will be referred to in this paper as *photoreactivation*.[3] The clear-cut and sweeping nature of photoreactivation is illustrated in figure 1.

Numerous workers have reported on the "antagonism" of various radiations to ultraviolet light (see review by Prát, 1936). However, the effects noted have usually been so small, and many experiments so undecisive, that their significance has been understandably overlooked. Perhaps the most pertinent work was that of Whitaker (1942), who showed that the ultraviolet-induced lengthening of the lag phase in *Fucus* eggs was counteracted in part by illumination with white light. Unfortunately, the effect was comparatively slight, and the phenomenon was apparently not investigated further.

That some ultraviolet-irradiated cells may recover if stored in suspension after irradiation has been observed by many (Hollaender and Emmons, 1941; Roberts and Aldous, 1949; see Kelner, 1949, for other references). The degree of recovery in stored suspensions has been relatively small, and since the possible reactivating effect of light from the room has not been controlled in such experiments, the data must be re-evaluated.

METHODS

Details of the method for the ultraviolet irradiation and the preparation of cells are found in a previous publication (Kelner, 1949), with the following additions for the bacteria studied:

Escherichia coli B/r (a strain chosen because it was used in genetic studies by Demerec and Latarjet, 1946) was grown in a synthetic liquid medium, M-9

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[1] Preliminary reports of some of these data given at the May, 1949, meetings of the Society of American Bacteriologists, and at the AEC Information Meeting for Biology and Medicine at Oak Ridge National Laboratory, in April, 1949.

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[3] The use of this term was suggested by Dr. Max Delbrück.

(the ammonium medium of Anderson, 1946) for 48 hours with aeration at 37 C. The grown cultures had reproducible titers of about 4.5×10^9 cells per ml and were diluted with saline to about 2×10^9 per ml before ultraviolet irradiation. Cultures were irradiated directly after removal from the incubator, or after preliminary chilling at 5 C for an hour. After irradiation the suspensions were immediately chilled and kept dark until treated with reactivating light. They could be kept chilled and dark for at least 8 hours without significant effect on subsequent reactivation.

Cells were irradiated with ultraviolet light at 20-cm distance from the source, a General Electric 15-watt germicidal lamp (intensity roughly about 50 ergs $\times$

Figure 1. A. Plate spread on the surface with *Streptomyces griseus* ATC 3326 conidia then irradiated with ultraviolet light. Following irradiation, the lid of the petri dish, with black tape in the pattern of an inverted T, was replaced, and the spores were illuminated with reactivating light. Dark survivors are seen in the shadow of the tape, and a mass of photoreactivated cells wherever visible light struck the cells *B* As in *A*, except plate was spread with *E. coli* B/r. Following irradiation, the petri dish lid, entirely covered with black tape except for a small square, was replaced, and cells illuminated with reactivating light. Dark survivors seen in shadow of tape, and photoreactivated cells in the image of tungsten filament. Visible light was projected on the square pattern through a projection lantern.

sec^{-1} $\times$ mm^{-2}). Ninety-five per cent of the ultraviolet radiation of this source was at 2,537 A.

For photoreactivation, cell suspensions were illuminated for 45 to 60 minutes in small glass tubes suspended in a glass-fronted water bath at 37 C. The reactivating light source was a 500-watt tungsten projection lamp (GE 500T20-120V) in a projection lantern with the bellows fully contracted. To obtain the highest light intensity, the cells were placed at the focal point of the beam. The source emitted radiations in the long ultraviolet as well as in the visible and infrared. A filter of 0.03 N aqueous CuCl$_2$ in a 3.2-cm deep cell was routinely used to absorb a large part of the infrared.[4] Suspensions were assayed for viable cells by spread-

[4] While the reactivating light is referred to in this paper as visible light, it should be remembered that long ultraviolet, as well as visible light, reached the cells.

ing 0.1 ml of suitable dilutions on the surface of plates containing Difco nutrient agar to which had been added 0.5 per cent NaCl, and 1:2,500,000 gentian violet to inhibit contaminants. Incubation was at 37 C in the dark, for 24 to 48 hours.

Complications due to unwanted photoreactivation of cells during handling, from light necessary for working, were avoided by illuminating the laboratory with yellow light only. A preliminary experiment showed that wave lengths longer than about 5,100 A did not cause appreciable photoreactivation. A convenient source of yellow light was a General Electric "gold" fluorescent bulb wrapped in amber gelatin filter sheets.

<div align="center">RESULTS</div>

Some of the terms used in this paper may need clarification. In an ultraviolet-irradiated suspension kept in the dark, the population consists of (a) viable cells, or the *dark survivors*, and (b) inactivated or non-colony-forming cells. If the irradiated suspension is treated with reactivating light, the population will consist of (a) *light survivors*, comprising the original dark survivors plus *photoreactivated* cells, and (b) inactivated cells which have not recovered despite

<div align="center">TABLE 1</div>
<div align="center">*Photoreactivation in four microbial species*</div>

	Streptomyces griseus	*Escherichia coli*	*Penicillium notatum*	*Saccharomyces cerevisiae*
Dark survival*	2.1×10^{-6}	4.5×10^{-6}	5.5×10^{-4}	1.0×10^{-5}
Light survival†	6.6×10^{-1}	1.2×10^{-1}	2.5×10^{-1}	1.0×10^{-3}

* Fraction of cells surviving in suspensions kept dark after ultraviolet irradiation.

† Fraction of cells surviving in suspensions illuminated with reactivating light after ultraviolet irradiation.

exposure to visible light. *Dark survival* is the number of dark survivors divided by the total number of cells before ultraviolet irradiation; *light survival* is the number of light survivors divided by the total number of cells before ultraviolet irradiation.

Generality of photoreactivation. Table 1 demonstrates photoreactivation in *Streptomyces griseus* ATC 3326, *Escherichia coli* B/r, *Penicillium notatum* 1951. B25, and *Saccharomyces cerevisiae*, a diploid strain received from Dr. C. Lindegren. All were irradiated in suspension with a dose of ultraviolet light giving the dark survival shown in table 1 and then reactivated with visible light, with a resulting increase in survival rate. The maximum degree of photoreactivation possible for *P. notatum* and *S. cerevisiae* may be greater than indicated, for these species were not studied intensively.

Photoreactivation in E. coli. As in *S. griseus* (Kelner, 1949) the degree of photoreactivation in *E. coli* was proportional to the time times the intensity of reactivating light, and the effect increased with rise in temperature, within limits, the Q_{10} between 20 and 40 C being about 3. Experiments with Wratten filters showed that the most effective wave lengths for photoreactivation were below 5,100 A. Slight recovery occurred at times in ultraviolet-irradiated sus-

pensions incubated at 37 C in the dark. The increase in survival rate, or dark recovery, was always slight, never exceeding in our experiments a value of 1.5- to 2-fold. Illumination of cells with visible light before ultraviolet irradiation had no effect on subsequent survival. Simultaneous irradiation of cells with ultraviolet and reactivating light, or use of strictly monochromatic ultraviolet light was not studied.

The dose-reduction ratio. Suspensions of *E. coli* irradiated with varying doses of ultraviolet light were subjected to the same amount of reactivating light (60 minutes of intense illumination at 37 C, as described under "Methods"). For each ultraviolet dose, an assay was made of the suspension before and after photoreactivation, and from these data, the dark-, and light-survival-ultraviolet-dose curves in figure 2 were plotted. It is clear that the two curves have similar shapes.

In table 2 the ultraviolet dose giving a specific dark survival (D) is compared with the ultraviolet dose giving numerically the same light survival (L). The doses were determined graphically from figure 2. As expected from the similarity in curves, the ratio L/D is remarkably constant throughout the whole range of ultraviolet dosages, averaging in this case 2.5. We will call L/D the "dose-reduction ratio."

Knowing the dark survival curve and the dose-reduction ratio for a given amount of reactivating light, one can predict the degree of photoreactivation after various ultraviolet doses. For example, if a culture of *E. coli* B/r is irradiated with an ultraviolet dose of 35 seconds, giving a dark survival of 1×10^{-2}, the light survival will be the same as the dark survival for the dose $\frac{35}{2.5}$, or 14 seconds, equaling a survival of 3.2×10^{-1}, or a 32-fold increase in survival due to photoreactivation.

Without implying anything as to the mechanism of photoreactivation, we may say that in *E. coli* B/r a given amount of reactivating light removes a constant percentage (60 per cent in the experiments described here) of the lethal effects of the ultraviolet dose.

The applicability of the dose-reduction principle to organisms other than *E. coli* B/r and the variation of the dose-reduction ratio with the amount of reactivating light were not studied.

Loss in ability to be photoreactivated. Whatever the primary effect of ultraviolet light, photoreactivation shows it is not immediately lethal to the cell. In cells incubated in the dark ,in a favorable medium after irradiation, when does the ultraviolet injury become irreversible?

An ultraviolet-irradiated suspension of *E. coli* (dark survival, 1.6×10^{-5}) was diluted 1:10 with sterile broth, then incubated at 37 C, with aeration in the dark. Samples were removed at intervals, diluted 1:10 with chilled saline to stop growth, and treated as follows: one portion of the sample was assayed immediately for viable cells; a second was assayed after being incubated 45 minutes at 37 C in the dark (furnishing data for the "dark" curve in figure 3); and a third was assayed after being illuminated with light 45 minutes at 37 C

(curve marked "light" in figure 3). These assays disclosed the degree of photo-reactivation after various periods of preincubation in the dark. There was no appreciable increase in cell count during the control incubation for 45 minutes
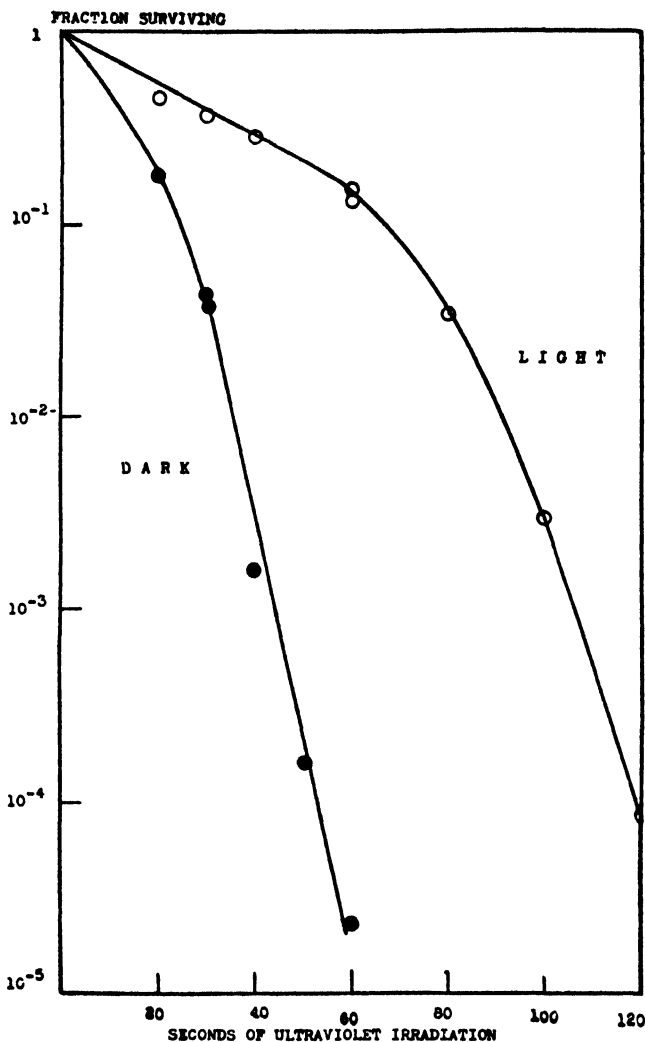
Figure 2. Ultraviolet dose-survival curves of *E. coli* B/r. Dark (black circles): suspension left in dark after irradiation. Light (open circles): suspensions reactivated with constant amount of visible light after ultraviolet-irradiation.

in the dark, except in the last (132-minute) sample, in which the count increased twofold, possibly because of division. The relative photoreactivation after various periods of preincubation is shown by the line of dashes in figure 3 and represents the quotient of the "light" divided by the "dark" assays.

Figure 3 shows that preincubation of ultraviolet-irradiated cells in nutrient

broth at 37 C in the dark results in an exponential loss in the ability to be photo-reactivated. After 2 hours, or at about the time the dark survivors start dividing, reactivating light has scarcely any effect. In other experiments, photoreactiv-ability disappeared after 3 hours of preincubation.

Cells incubated in saline prior to visible light illumination also lost their ability to be photoreactivated, but at a much slower rate. After 2.4 hours of preincubation in saline, instead of broth, in one experiment, the suspension recovered 113-fold after visible light illumination, as compared to a 4,100-fold recovery with no preincubation. Nutrient broth is thus not essential for the changes leading to the loss in ability to be photoreactivated, although it does accelerate the loss. A direct relation of metabolism to loss in recoverability is

TABLE 2

Dose-reduction ratio of E. coli

SURVIVAL RATE	ULTRAVIOLET DOSE (SECONDS)		DOSE-REDUCTION RATIO (L/D)
	Light (L)	Dark (D)	
6.3×10^{-1}	15	6	2.5
3.2×10^{-1}	36	15	2.4
1.0×10^{-1}	67	24	2.8
5.0×10^{-2}	76	28	2.7
1.0×10^{-2}	91	36	2.5
5.0×10^{-3}	96	38	2.5
1.0×10^{-3}	106	44	2.4
6.3×10^{-4}	108	46	2.4
3.2×10^{-4}	112	48	2.3
8.9×10^{-5}	120	53	2.3
Average..............			2.5

L—Ultraviolet-irradiated suspension treated with reactivating light.
D—Ultraviolet-irradiated suspension kept dark.

not excluded by the saline experiment, since sufficient reserve foods may have been present in cells to allow considerable metabolic activity during incubation.

Cells kept dark and cold after ultraviolet irradiation retain their photoreactiv-ability for many hours. However, under otherwise favorable conditions there is a short period only, 2 or 3 hours, during which the ultraviolet-induced lethal processes can be reversed by light, even in part.

Genetic studies. Ultraviolet radiation of 2,537 A wave length is one of the most powerful mutagenic agents known. An important consequence of the dis-covery of photoreactivation may be the ability ultimately to relate the mutagenic action to some precisely characterized enzyme system or compound in the cell.

The experiments described here were designed to answer the question, Are induced mutants present among photoreactivated cells? If so, are they present at a frequency of the same order of magnitude as among the dark survivors?

Techniques for the quantitative study of ultraviolet-induced mutation in
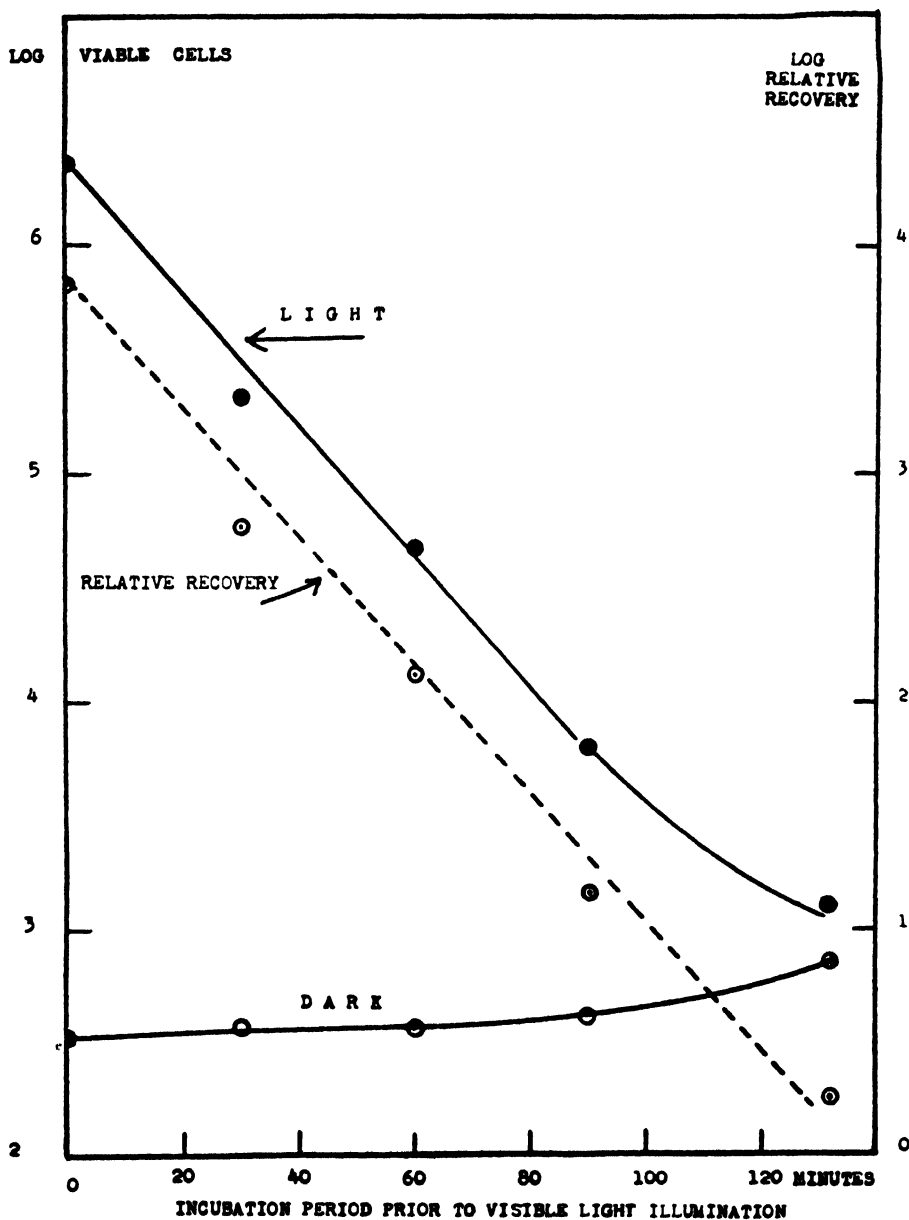
Figure 3. Loss in ability to be photoreactivated, with incubation of ultraviolet-irradiated *E. coli* B/r cells in the dark in nutrient broth prior to treatment with visible light. Dark: assay in suspensions incubated in dark after ultraviolet irradiation. Light: assay of suspensions treated with constant amount of reactivating light after various periods of preincubation. Relative recovery: the quotient of the light divided by the dark assays. Note exponential loss in ability to recover.

E. coli B/r from sensitivity to resistance to coliphage T-1 have been worked out by Demerec and Latarjet (1946). They have distinguished two types of phenotypic expression of the mutant—(1) "zero-point" mutants, or those whose mutant character is phenotypically expressed within a few minutes after irradiation, and (2) "delayed" mutants, which express their mutant character only after incubation on nutrient medium for a variable length of time, and, perhaps, only after undergoing one or two divisions. The delayed mutants are always present at a greater frequency than the zero-point mutants for any given dose of ultraviolet light. Demerec and Latarjet (1946) studied the frequency of induced mutants in what we have called the dark survivors, except that they probably did not take precautions to exclude visible light in their experiments.

Induced zero-point mutants in photoreactivated cells. A culture of *E. coli* B/r diluted to a titer of about 2×10^9 per ml, as described previously, was divided into several parts. In one, the frequency of spontaneous phage-resistant mutants was determined by spreading 0.1-ml portions of appropriate dilutions of the suspension on nutrient agar plates previously coated with 1 to 2×10^9 T-1 phage particles each. The colonies appearing after 48 hours of incubation in the dark represented resistant mutants, and from the total number of cells seeded to the plate (as determined by suitable assay), the frequency of spontaneous phage-resistant mutants could be calculated. In the suspensions used the frequency varied from 5 per 10^8 to 85 per 10^8.

A second portion of the suspension was irradiated with ultraviolet light as indicated in table 3, an assay made for dark survival, and 0.1-ml portions of the undiluted suspension spread on phage-coated plates to determine the number of resistant mutants among the dark survivors contained in this volume of ultraviolet-irradiated suspension. No resistant mutants were found on any of the plates, as could be expected from the small number (900 to 76,000) of dark survivors in 0.1 ml of irradiated suspension.

At the same time, a portion of the ultraviolet-irradiated suspension was photoreactivated as described previously, an assay made for total viable cells, and 0.1-ml portions of undiluted suspension spread on phage-coated plates. The phage-resistant colonies on the latter plates could come from three sources, (1) spontaneous mutants which had been inactivated by ultraviolet light and reactivated by visible light, (2) spontaneous and induced mutants in the dark survivors included in the 0.1 ml of photoreactivated suspension seeded to each plate, and (3) induced mutants from photoreactivated cells. In our experiments the correction for (2) was zero. After correction for (1) the frequency of induced mutants in photoreactivated cells was calculated and is shown for 6 experiments in table 3.

It is seen that, at the ultraviolet dosages used, induced zero-point mutants are present among photoreactivated cells, but at a very low frequency.

Demerec and Latarjet (1946) had found that at ultraviolet doses comparable to those used by us, the frequency of zero-point mutants (in dark survivors) was of the order of 10,000 per 10^8, or over 500 times higher than we found in photoreactivated cells. As a further check on our results, an ultraviolet-irradi-

ated suspension was concentrated by centrifugation, and then the induced zero-point mutants were determined, after the suspension was incubated 45 minutes in the dark at 37 C as the control, and for comparison after photoreactivating as usual. The dark survival in this experiment was 1.2×10^{-4}, the recovery after photoreactivation 1,200-fold. The frequency of induced zero-point mutants among the dark survivors was 2,900 per 10^8, a figure in fair agreement with the data of Demerec and Latarjet (1946); the frequency in the photoreactivated cells was as expected only 11 per 10^8.

We may infer conservatively that for the ultraviolet dose used, and the techniques employed, induced zero-point mutants are present at a much smaller frequency among photoreactivated cells than in dark survivors.

Delayed mutants. The methods for determining delayed mutants in *E. coli* B/r were adapted from those of Demerec (1946), Demerec and Latarjet (1946), and Beale, (1948). One-tenth ml of suitable dilutions of nonirradiated, ultraviolet-irradiated, and photoreactivated *E. coli* B/r suspensions were spread on

TABLE 3

Induced zero-point mutants in photoreactivated E. coli

ULTRAVIOLET DOSE	DARK SURVIVAL*	LIGHT SURVIVAL†	INDUCED MUTANTS PER 10^8 PHOTOREACTIVATED CELLS
sec			
65	1.2×10^{-5}	1.3×10^{-1}	21
65	3.1×10^{-5}	2.0×10^{-1}	0
65	4.5×10^{-6}	1.2×10^{-1}	6
55	3.8×10^{-4}	3.3×10^{-1}	13
55	6.7×10^{-5}	2.5×10^{-1}	16
55	4.3×10^{-5}	2.8×10^{-1}	12

* Fraction of cells surviving in suspensions kept dark after ultraviolet irradiation.

† Fraction of cells surviving in suspensions illuminated with reactivating light after ultraviolet irradiation.

the surface of nutrient agar plates. After various periods, up to 5 to 6 hours, of incubation at 37 C in the dark, plates were sprayed with 1 to 3×10^9 T-1 phage particles in the form of a fine mist of the phage lysate. The plates were then incubated further for 48 hours, and the phage-resistant colonies were counted. The average total number of viable cells on the plate when sprayed with phage was determined by assaying the bacteria washed off of comparable plates with saline (Beale, 1948). From these data, the mutation rate from sensitivity to resistance during various increments of the first 5 to 6 hours of incubation was calculated.

As Demerec (1946) has shown, the mutation rate is highest during the first few hours of incubation, presumably during the first few divisions of irradiated cells, then drops off to the spontaneous rate. We therefore felt that measurement of the mutation rate during the first 5 hours of incubation would give us a sensitive measure of delayed mutation and would avoid complications due to spraying phage on older plates that had excessive numbers of bacteria and the attendant problems of incomplete adsorption of phage and the like.

In table 4 are shown delayed mutation rates in photoreactivated cells; and as controls, in dark survivors and nonirradiated cells. Complications due to the presence of dark survivors among photoreactivated cells were avoided by seeding plates with high enough dilutions of photoreactivated suspensions to dilute the dark survivors out. The frequency of delayed mutation, calculated

TABLE 4

Effect of photoreactivation on delayed mutation in E. coli B/r

PREINCUBATION BEFORE SPRAYING WITH PHAGE	TOTAL CELLS ON PLATE	B/r/1* (AVG. NO. PER PLATE)	INCREMENT PERIOD	INCREMENT IN		DELAYED MUTATION RATE†
				Total cells	B/r/1*	
(Expt. 59-A)‡ Control—nonirradiated						
hr			*hr*			
0	8.8×10^4	0	—	—	—	—
3	2.1×10^6	0.75	0–3	2.0×10^6	0.75	37
4	2.2×10^7	0.5	3–4	2.0×10^7	0	0
5.8	7.0×10^8	3	4–5.8	6.8×10^8	2.5	0.4
(Expt. 59-A)‡ Photoreactivated cells						
0	8.8×10^4	0	—	—	—	—
3	4.1×10^5	4.5	0–3	3.2×10^5	4.5	1,400
4	4.1×10^6	14	3–4	3.7×10^6	9.5	260
5.1	3.3×10^7	21	4–5.1	2.9×10^7	7	24
(Expt. 61-A)§ Dark survivors						
0	6.4×10^4	0	—	—	—	—
3.1	5.3×10^4	0.25	0 –3.1	—	—	—
4.1	4.8×10^5	1.5	3.1–4.1	4.3×10^5	1.25	290
5.0	3.8×10^6	8	4.1–5	3.3×10^6	6.5	200
(Expt. 61-A)§ Photoreactivated cells						
0	1.4×10^5	0	—	—	—	—
3.1	6.0×10^5	1.5	0 –3.1	4.6×10^5	1.5	330
4.1	6.9×10^6	22	3.1–4.1	6.3×10^6	20.5	326
5.0	5.5×10^7	31	4.1–5	4.8×10^7	9	19

* B/r/1—*E. coli* B/r mutant resistant to phage T-1.

† Number of B/r/1 mutants per 10^8 new cells that appeared during the increment period indicated.

‡ In expt. 59-A the dark survival equaled 6.7×10^{-5}; light survival, 2.5×10^{-1}.

§ In expt. 61-A the dark survival equaled 3.8×10^{-4}; light survival, 3.3×10^{-1}.

on the basis of the number of cells seeded to the plate (see Demerec and Latarjet, 1946) was determined for photoreactivated suspensions in experiments not shown in table 4 and showed the same order of magnitude for both dark survivors and photoreactivated cells, 1 to 2 $\times$ 10^4 per 10^8. This figure agrees fairly well with the values (about 7 $\times$ 10^4 per 10^8) given by Demerec and Latarjet (1946) for comparable doses of ultraviolet light.

The data in table 4 show that, at the ultraviolet dosages used by us, the

mutation rates for delayed mutants are of the same high order of magnitude in both dark survivors and photoreactivated cells.

The experiments here described show conclusively that induced mutants appear in photoreactivated cells, but do not decisively answer the question of whether reactivating light has an effect on mutation comparable to that which it has on the lethal effects of ultraviolet light.

DISCUSSION

The demonstration of photoreactivation in several microbial species increases the probability that light reactivation will occur in most microorganisms and, perhaps, in higher forms as well. If this is so, it follows that the cellular compounds concerned in the recovery process are common to most cells. The challenge that confronts us is to determine the nature of these compounds and the mechanisms involved in photoreactivation.

The dose-reduction principle should furnish a basis upon which to compare different effects of ultraviolet light. For example, on first glance, our result with mutations appears inconsistent in that it implies (a) that light reactivation causes a pronounced reduction in mutation frequency (data on zero-point mutations) or (b) that it has no effect (data on delayed mutation). However, the results are consistent if we assume the dose-reduction principle to hold for mutagenesis as well as for lethal effects. This would mean that the mutation frequency in light survivors is equal to that found in the dark survivors at an ultraviolet dose equal to the actual dose used, divided by the dose-reduction ratio. In the dose-mutation frequency curves shown by Demerec and Latarjet (1946, figure 1), at the points corresponding to the ultraviolet doses used by us (giving a dark survival of about 1×10^{-4} to 1×10^{-5}), the curve for delayed mutants rises slowly, whereas the curve for zero-point mutants rises rapidly. If the reactivating light reduces the effective ultraviolet light by a constant ratio, as assumed by the dose-reduction principle, or, in other words, pushes the mutation frequency back on the curve, we should expect a far greater reduction in the mutation of zero-points than for delayed mutants. Taking into consideration the variation in our technique from that of Demerec and Latarjet (1946), our finding that zero-point mutation is more strongly reduced by reactivating light than is delayed mutation is consistent with the hypothesis that the dose-reduction principle holds for mutagenesis. According to the shape of the dose-mutation frequency curve at the ultraviolet dose used, reactivating light should have either no effect on mutation frequency (horizontal curve), or cause a reduction (rising curve) or increase (falling curve) in frequency.

Consistency of data with the dose-reduction principle is of course insufficient proof that the latter is correct for mutagenesis. Moreover, since the significance of zero-point and delayed mutation is currently quite uncertain (see Demerec and Latarjet, 1946; Newcombe, 1948) other explanations for our data may prove more accurate.

There can be no doubt, however, that the solution of not only the problem of ultraviolet mutagenesis, but mutagenesis in general, will be considerably advanced by the elucidation of the mechanism of photoreactivation.

SUMMARY

Visible light of wave lengths under 5,100 A will cause the recovery of microbial cells from ultraviolet-induced injury which would otherwise be fatal. Light-induced recovery, or photoreactivation, occurs in at least four diverse species, *Escherichia coli* B/r, *Streptomyces griseus* ATC 3326, *Penicillium notatum*, and *Saccharomyces cerevisiae*.

In *E. coli* B/r the ultraviolet dose-survival curves for suspensions kept dark after irradiation, and for suspensions photoreactivated, have in general similar shapes. From the similarity in shape of the curves there was evolved the *dose-reduction principle*—the effect of a constant amount of reactivating light on survival in a suspension irradiated with varying amounts of ultraviolet light is the same as if it decreased the effective ultraviolet dose by a constant factor. For *E. coli* B/r this means that the amount of reactivating light used in one experiment reduced the dose of ultraviolet light effective in killing cells by 60 per cent.

E. coli B/r cells incubated in broth at 37 C, in the dark, after ultraviolet irradiation, lost their ability to be photoreactivated. The ability to recover decreased exponentially with incubation time, becoming zero after 2 to 3 hours of incubation.

Induced mutants occur in photoreactivated *E. coli* B/r cells. At the ultraviolet doses used, reactivating light apparently reduced the frequency of mutants characterized by phenotypic expression within a few minutes after irradiation (zero-point mutants) but had little or no effect on mutants characterized by phenotypic expression only after a prolonged period of incubation. The possible significance of these data in the light of the dose-reduction principle is discussed.

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THE VITAMIN REQUIREMENTS OF STENOTHERMOPHILIC AEROBIC SPOROGENOUS BACILLI

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The study of thermobiosis in bacteria might be facilitated by the employment of a satisfactory simple medium, in order to maintain the normal dynamic state of growing cells, as suggested by Rahn and Schroeder (1941). Gaughran (1947) stated that the field of essential nutrilites for these organisms has been untouched. Accordingly a study of vitamin requirements was the subject of this investigation. Gordon (1947) expressed the opinion that the ability to grow well at 65 C is of taxonomic importance for the stenothermophiles. All studies were therefore made at the classical temperature of 55 C and at 65 C.

MATERIALS AND METHODS

Cultures. Twelve cultures, considered to be representative, were studied. All grew well in nutrient broth and on nutrient agar at 55 C and 65 C, but failed to show visible growth on nutrient agar slants after 3 weeks at 35 C. All were sporogenous, facultative, gram-positive to gram-variable rods. Culture 10 was a University of Maryland stock strain; NRS 91, *Bacillus stearothermophilus*, was obtained from the American Type Culture Collection; cultures 26, 1356, 1492, 1503, 1518, 1792, 1805, 4103, and 4298 were "flat sour organisms" obtained from the National Canners Association.

Glassware. Pyrex glassware, aluminum caps, and glass-distilled water were used throughout. All glassware was chemically cleaned. Nutrient solutions were filtered through porcelain funnels (Selas 02); concentrated vitamin solutions were filtered through sintered glass (Corning UF). The filters were cleaned with aqua regia.

Incubation. A double-walled air incubator was used for cultures at 55 C ± 1. Temperature was taken from a thermometer immersed in water. A covered water bath was used for incubation at 65 C ± 0.25.

Preparation of casein hydrolyzate medium. Casein hydrolyzate medium was employed since it was found to support good growth when suitably supplemented. The basal medium was prepared by boiling 1 mg L-cystine, 10 mg DL-tryptophan, 100 mg NaCl, and 0.5 g vitamin-free casein hydrolyzate[2] in 95 ml water. After filtration, 2.5 ml of a sterile 20 per cent solution of glucose (filtered) and 5 ml of a sterile 10 per cent solution of K_2HPO_4 (autoclaved) were added. After the addition of the vitamins, the medium was pipetted into sterile 16-by-125-mm tubes, in 5-ml amounts. The final pH of the medium was 7.2 to 7.4.

Preparation of inoculum. Cultures were grown 20 to 24 hours at 55 C in centrifuge tubes containing medium of the following composition: trypticase, 1 per cent; yeast extract, 0.2 per cent; NaCl, 0.5 per cent; glucose, 0.5 per cent; K_2HPO_4, 0.5 per cent; final pH, 7.4. (Sterile glucose and phosphate solutions were added after autoclave sterilization of the rest of the medium.) The cells were harvested by centrifugation and prepared for use by washing twice with 0.9 per cent NaCl before final resuspension. Such a cell suspension contained relatively old cells and practically no spores. Plate counts as determined in trypticase yeast extract glucose agar at 55 C showed that one drop of the cell suspension contained from 350 to 10,000 viable cells per drop.

Screening of vitamins. The following vitamins, in the ranges of concentrations per ml indicated, were added to the basal medium, singly and in many combinations: pteroylglutamic acid and biotin (free acid), 0.1 to 1 μg; i-inositol and p-aminobenzoic acid, 1 μg to 1 mg; choline, riboflavin, pyridoxine, calcium-D-pantothenate, niacin, and thiamine, 1 to 10 μg. The tubes of complete medium were preheated to 55 C and 65 C and inoculated rapidly with a drop of the cell suspension. Four subsequent 24-hour serial transfers were made with a 4-mm loop. Growth response was detected by turbidity measurement. The approximate final pH of the cultures was determined colorimetrically. Graded amounts of the essential vitamins were supplied the organisms, and the growth responses observed.

Microscopic examinations. Stained preparations (Giemsa and Gram) of all cultures were frequently examined to observe differences in spore yield, morphology, and stainability engendered by changes in composition of the medium and in incubation temperature.

RESULTS AND DISCUSSION

A medium was considered productive of good growth only if it supported at least 50 per cent as much growth as trypticase yeast extract glucose phosphate broth. Similarly, since a prominent feature of many thermophiles is the rapid dissimilation of glucose with the consequent production of abundant acid (Hansen, 1933), a medium was considered suitable only if the final pH was about 4.5. This is applicable to all cultures in this study except 1805 and 4103. These do not ferment glucose rapidly in the casein hydrolyzate medium employed.

Biotin, niacin, and thiamine were the only vitamins found essential for continued growth of the 12 cultures, at 55 C and 65 C, in the medium used. As is shown in table 1, one group of 5 cultures required all three vitamins, another group of 5 cultures required only biotin and niacin, and a third group of 2 cultures required only biotin. It is seen, however, that all organisms are stimulated by the three vitamins together. Niacin and thiamine, 1 μg each per ml, and biotin, 0.04 μg per ml, were found to be the optimum concentrations. Vitamin concentrations of 10 times these amounts did not improve growth. Since considerable similarity was found among the organisms in each group, average replicate turbidities are shown in table 1, indicating the responses of the organisms to graded amounts of the three vitamins and in the natural medium mentioned

above. No temperature-correlated difference in vitamin requirements was found with any culture, with the exception of organism 4103. This organism, in some experiments, grew well at 55 C in the absence of biotin, although it uniformly

TABLE 1

Growth response of 12 stenothermophiles to varying amounts of biotin, niacin, and thiamine in casein hydrolyzate medium at 55 C and 65 C

ORGANISMS	TEM-PERA-TURE	Biotin: 0 / Niacin: 1.0 / Thiamine: 1.0	0.000004 / 1.0 / 1.0	0.00004 / 1.0 / 1.0	0.04 / 0 / 1.0	0.04 / 0.001 / 1.0	0.04 / 0.01 / 1.0	0.04 / 0.1 / 1.0	0.04 / 1.0 / 0	0.04 / 1.0 / 0.00001	0.04 / 1.0 / 0.001	0.04 / 1.0 / 0.01	0.04‡ / 1.0‡ / 1.0‡	TRYPTICASE YEAST EXTRACT GLUCOSE PHOSPHATE BROTH
Group 1 10, 1356, 1503, 1518, NRS 91	55	0*	2*	20*	0	10	21	45	0	20	43	49	52	75
	65	0	0	19	0	2	17	42	0	20	34	42	47	70
Final pH		7.4	7.4	5.0	7.4	7.4	7.0	5.0	7.4	6.6	5.0	5.0	4.4	4.2
Group 2 26, 1492, 1792, 2156, 4298	55	0	2	34	0	13	25	44	40	40	44	50	52	75
	65	0	0	34	0	3	19	41	40	40	42	44	49	70
Final pH		7 4	7.4	5.0	7.4	7.4	7.0	5.0	5 0	5.0	5 0	4 4	4.4	4.2
Group 3 1805, 4103	55	0†	0	21	32	41	41	41	32	32	35	35	44	80
	65	0	0	17	32	36	36	36	32	32	34	34	40	75
Final pH		7.4	7.4	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7 0	7.0	7.0	6.6

Concentration, μg per ml

* Figures represent the average replicate turbidities of serial 24-hour transfers of all organisms in each group, as measured with the Fisher electrophotometer, AC model, using the 425 B filter. Figures obtained by subtracting light transmittance from 100.

† Organism 4103 variable. See text.

‡ Optimal medium.

required biotin at 65 C. This feature is not stable, and special attention to purity and phase of culture and cleanness of glassware did not reveal an explanation for this finding. When supplied suboptimum amounts of vitamins, the fermentative power of the organism was lessened as indicated by higher final pH, although growth was substantial.

None of the casein hydrolyzate media used supported more than about 60 per cent as much growth as the trypticase yeast extract medium.

Microscopic examination revealed that the spore yield, which is consistently low for the stenothermophiles, is about the same in 24 hours in natural and casein hydrolyzate media. Prolonged incubation in either medium yields more spores, although fewer are produced at the higher temperature. At the higher temperature, also, the cells stain less evenly, and show greater variation in size and shape.

It appears that even for the stenothermophiles, a temperature of 65 C is considerably less satisfactory for growth than 55 C. The relatively high concentration of vitamins required for usual growth of these organisms suggests that a rapid rate of heat inactivation of enzyme or intermediate compound occurs even with the thermophiles, and that unless the medium provides a reasonable excess, growth and fermentative ability are suppressed.

Bacillus coagulans, a eurithermophile, has been found to require the same three vitamins in the same concentrations for optimum growth (Cleverdon, Pelczar, Doetsch, 1949).

SUMMARY

Twelve stenothermophiles grew well in serial transfer, at 55 C and 65 C, in a casein hydrolyzate medium when supplied biotin, 0.04 μg per ml, and niacin and thiamine each 1 μg per ml. No significant differences in vitamin requirements at the two temperatures were found. At the higher temperature, growth was less abundant, the cells showed greater variation in morphology, and spore production was depressed, in both natural and casein hydrolyzate media.

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AN ANTIPHAGE AGENT ISOLATED FROM ASPERGILLUS SP.

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The ability of some known antibiotics such as streptothricin, streptomycin, and clavacin to prevent bacteriophage action has been demonstrated (Jones, 1945). Schatz and Plager (1948), working with an actinomycete that demonstrated antiphage activity, extracted a material that was capable of weakly inhibiting a rodent-paralyzing virus (MM), but it appeared to be too toxic for further study. We have isolated a substance from *Aspergillus* sp. that was capable of inhibiting *Staphylococcus aureus* 209 bacteriophage, but exhibited no antiviral activity *in vivo* when tested against poliomyelitis (MM) and influenza (PR-8-A) infections in mice. This report is submitted for its intrinsic value as a method of testing and isolating an antiphage agent.

The isolation and testing of the antiphage-producing organisms were accomplished by techniques similar to those described by Jones and Schatz (1946). Various soil samples, used as a source of antiphage-producing organisms, were cultured on nutrient agar plates for 10 days at room temperature. The plates were then sprayed with semisolid nutrient agar (0.7 per cent agar), seeded with 1 per cent of a 24-hour *S. aureus* 209 culture and a 0.1 per cent *S. aureus* phage suspension (6 $\times$ 10^8 phage particles per ml), and incubated at 37 C for 16 hours. The presence of *S. aureus* growth in the sprayed layer surrounding a soil colony indicated inhibition of the bacteriophage.

Several molds, actinomycetes, and bacteria antagonistic to *S. aureus* phage were isolated. An *Aspergillus*, designated culture H-3, demonstrated greater phage-suppressing ability than other isolates and was selected for further study.

Culture H-3 produced the antagonistic factor in several liquid media. A dextrin corn steep medium gave the highest yield in shake flasks and has been used successfully in 10-gallon and 100-gallon fermenters. Potencies ranging from 20 to 200 units per ml of beer have been obtained.

To test quantitatively the activities of fermentation beers and subsequent extraction samples a plate assay was developed in which zones of phage inhibition were measured. One-fourth-inch paper disks were dipped into the solutions to be tested and placed on nutrient agar plates composed of a 10-ml base layer plus a 5-ml semisolid surface layer seeded with 1 per cent 24-hour *S. aureus* culture and 0.1 per cent *S. aureus* phage suspension. Plates were incubated for 16 hours at 37 C to allow for zone development. A solution containing enough antibiotic H-3 to give a zone of *S. aureus* growth 8 mm in diameter was defined as having a potency of 1 unit per ml. A preparation containing 40 units per mg

was subsequently prepared as a standard. This preparation was diluted daily to afford solutions of 1.25 to 10 units per ml. This range in concentration represented a total zone diameter difference of about 10 mm on the standard curve.

Extraction of the neutral filtered beer with chloroform gave a brownish-yellow gum which was dried to a powder under vacuum. It was soluble in most organic solvents with the exception of the saturated hydrocarbons. The solubility of antibiotic H-3 in water decreased as it was purified. It was fairly stable to heat and acid but was quickly destroyed by alkali.
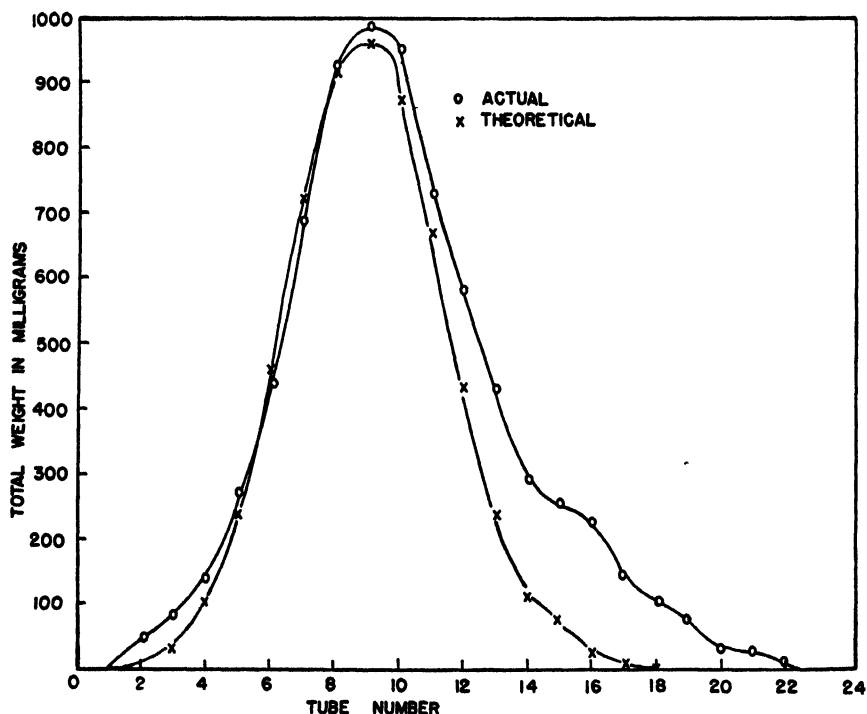
Figure 1. Countercurrent distribution of a sample of H-3.

Although ultimate purity has not been achieved at this time, a 400-fold purification from the beer solids has been obtained by means of countercurrent distribution. The dried material from the neutral chloroform extraction of the beer was dissolved in ethyl ether, and the resulting solution·was shaken several times with a 30 per cent methanol and 70 per cent water mixture. The ethereal phases, when dried under vacuum, afforded a powder the activity of which was from one to five times greater than that of the starting material.

A sample of antibiotic H-3 prepared by this procedure contained 270 units ' per mg and demonstrated little antibacterial or antifungal activity. In a broth dilution test, *Streptococcus faecalis* 6057 was inhibited at 300 μg per ml, *Bacillus subtilis* at 100 μg per ml, and *S. aureus* at 30 μg per ml. A concentration of 300 μg per ml did not inhibit *Escherichia coli* 26, *Salmonella schottmuelleri* 9149,

Salmonella typhosa 167, *Klebsiella pneumoniae* PCI-602, *Brucella bronchiseptica* B-140, and *Mycobacterium tuberculosis* var. *hominis* 607. The fungal pathogens of man were not inhibited by the preparation in concentrations of less than 100 μg per ml when tested by an agar dilution method.

This preparation had a subcutaneous LD_{50} of about 9.6 mg per mouse. However, doses of 3.0 mg per mouse given subcutaneously twice daily for $3\frac{1}{2}$ days to mice infected with either poliomyelitis (MM) or influenza (PR-8-A) hastened death. One-mg doses gave no protection.

The limiting factor in the further purification of antibiotic H-3 through solvent distribution was its insolubility in water. A solvent system, analogous to that of Marshak *et al.* (1947), prepared by mixing equal volumes of 20 per cent cyclohexane in benzene and 10 per cent water in methanol, was used to obtain a preparation of antibiotic H-3 having a potency of 800 to 1,000 units per mg. Figure 1 illustrates a 24-tube distribution of 7 grams of a preparation of antibiotic H-3, having a potency of 450 units per mg, using 95 ml of each solvent phase. The material in tube 9 had an activity of about 1,000 units per mg. Conformity to the theoretical curve was good, and a subsequent distribution of 200 mg of fraction 9, using 10 ml of each phase, through 24 tubes gave a curve similar to that in figure 1. This would indicate that the material was close to maximum purity; however, all attempts at crystallization of the material in fraction 9 have so far failed. Hydrolysis of a portion of this fraction by the method of Sanford and Humoller (1947) followed by paper strip chromatography of the hydrolyzate indicated the presence of three ninhydrin-positive zones. In view of the limited amount of material available these zones were not identified.

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MICROORGANISMS IN THE INTESTINAL TRACT OF NORMAL CHICKENS[1]

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One of the objectives of studies on the intestinal flora of animals is to elucidate any interrelationships that may exist between the microorganisms and the host. Recently there has been renewed interest in the significance of the intestinal flora in relation to vitamin biosynthesis. These studies have been reviewed in detail by several authors (Najjar and Barrett, 1945; Elvehjem, 1946, 1948; Johansson and Sarles, 1949).

There have been few studies of the intestinal flora of normal chickens. Kern (1897) studied the intestinal microflora of the stomach and intestinal contents of 22 species of birds. He concluded that the following species were obligate intestinal forms: *"Bacterium coli commune," "Bacillus vegetus," "Pseudomonas granulata," "Bacillus defessus,"* and *"Bacterium verrucosum."* Kern believed that there were no obligate stomach species, that the stomach flora was merely a reflection of the food eaten, and hence was variable. A few years later, Rahner (1901) studied the microflora of the contents of various levels of the intestinal tract of chickens at various ages. He reported that *"B. coli gallinarum"* first appeared in two-day-old chicks whereas gram-positive rods and cocci appeared only in chicks four to five days of age. Rahner concluded that *"B. coli"* was the only obligate intestinal form. He did not detect any anaerobes. King (1905) also studied the intestinal microflora of different levels of the intestinal tract of chickens. His results were in agreement with those of Rahner in that *"B. coli"* was found in small numbers, if at all, in the duodenum but in the lower levels of the tract it was present in large numbers, and reached a maximum in the cecum. Gage (1911) reported that *"B. coli"* made up 60 per cent of the intestinal flora, whereas gram-positive cocci were found to constitute 30 per cent of the flora. He made the interesting observation that the ceca of newly hatched chicks were filled with gas, but was unable to isolate any anaerobes. Menes and Rochlin (1929) studied the intestinal microflora of hens, geese, and turkeys. They concluded that the flora was identical at all levels of the tract. The predominating inhabitants were *Escherichia acidi-lacti, Streptococcus faecalis,* and *Lactobacillus beijerincki.* Emmel (1930) reported that 50 per cent of the flora of two-week-old chicks and hens was made up of *Escherichia coli* and *E. coli* var. *communior.* He also detected considerable numbers of aerobic spore formers, but very few anaerobes.

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These early studies did not establish on a quantitative basis the kinds and numbers of microorganisms that make up the so-called normal intestinal flora of the chicken at the various levels of the intestinal tract. They did not provide the information needed as a foundation for combined studies by nutritionists and microbiologists.

The results of an investigation of the influence of various carbohydrates on the numbers and kinds of microorganisms in the intestinal tract of the chicken have been published by Johansson *et al.* (1948). The changes produced in the cecal flora of chicks infected with *Eimeria tenella* have been reported by Johansson and Sarles (1948). These studies showed again the need for knowledge of the normal flora of normal chickens.

The work reported in this paper represents an attempt to determine the kinds and numbers of certain groups of bacteria present at various levels of the intestinal tract of the normal chicken. It is hoped that this information will help to establish a foundation for future studies on the biological significance of the intestinal flora of the chicken.

METHODS

Animals and rations. The chickens used throughout the course of this work were the offspring of New Hampshire males crossed with Single Comb White Leghorn females. After the chicks hatched they were kept without food or water for 24 hours. The chicks were than placed in wire battery cages and given food and water *ad libitum.*

The initial ration used was starter mash no. 45 of Halpin *et al.* (1944). At the age of 10 weeks this ration was supplemented with a mixture of corn, wheat, and oats made up so as to supply 3 parts of corn, 2 parts of wheat, and 1 part of oats. At maturity, all the chickens were placed on a slightly different grain mash (Robblee *et al.*, 1948).

Two chicks were picked at random from the group for sampling at 3- to 4-day intervals up to the age of 45 days; at 7- to 14-day intervals up to the age of 30 weeks; and at irregular intervals thereafter up to the age of 1 year. In addition, 20-day-old chick embryos and chicks that had just hatched and had not yet been given food or water were also sampled.

Preparation of samples. Each chick was killed by decapitation and the intestinal tract from the gizzard to the cloaca was exposed. The specific segments to be sampled were set off by ligatures. The segments of the tract sampled were the duodenum, the ileum (approximately 6 inches of the middle of the small intestine), the cecal pouches, and the colon. The contents of each segment were squeezed under aseptic conditions into sterile petri dishes, which were immediately refrigerated. The time required between the killing of the chicken and the refrigeration of all samples varied between 5 to 10 minutes.

When all samples had been obtained, each was thoroughly mixed, and a 0.5-gram sample was weighed out on a piece of waxed paper. With very young chicks it was often impossible to obtain half-gram samples and the amount available had to be weighed and used. The weighed sample was thoroughly mixed in a 6-ounce bottle containing 49.5 ml of sterile tap water and a 6- to 8-mm layer of

small glass beads. Occasional samples were mixed for various lengths of time in sterilized stainless steel containers of a Waring blender. From these 1:100 dilutions, serial dilutions up to 10^8 were made in sterile tap water. From these dilutions inoculations were made as rapidly as possible into the tubes and petri dishes required for each cultural procedure.

Media and cultural procedures. (a) For aerobic agar plate counts the following medium was used: tryptone (Difco) 0.5 per cent, yeast extract (Difco) 0.3 per cent, glucose 0.5 per cent, and agar 1.5 per cent. The medium was adjusted to pH 6.8 to 7.0.

(b) For anaerobic agar plate counts the same medium as in (a) was used. Anaerobic conditions were obtained by incubating the plates in modified McIntosh and Fildes jars with a 100 per cent hydrogen atmosphere.

(c) Eosin methylene blue agar (Difco) plates were used for coliform plate counts.

(d) Potato glucose agar acidified to pH 3.5 was used for yeast plate counts (*Standard Methods for the Examination of Dairy Products*, 1941).

(e) "SF" broth of Hajna and Perry (1943) was used for dilution counts of enterococci.

(f) Carrot liver agar "shake" tubes (Garey *et al.*, 1941) were used to enumerate lactic acid bacteria.

(g) For spore counts, the 1:100 dilution of each sample was heated in a water bath at 80 C for 11 minutes and then rapidly cooled. From this heat-shocked sample suitable dilutions were made and agar plates of medium "a" (tryptone glucose yeast extract agar) were prepared. A duplicate series of plates of the same medium was also prepared and incubated anaerobically in a 100 per cent hydrogen atmosphere.

All the plates in cultural procedures (a), (b), (c), (d), and (g) were prepared in duplicate by the usual "pour" plate method. The "shake" tubes in cultural procedure (f) were also prepared in duplicate. The "SF" broth cultures in procedure (e) were prepared by using 5 tubes per dilution. The "SF" broth cultures were incubated at 45 C whereas the potato glucose agar plates were incubated at room temperature (20 to 25 C). The remaining cultures, procedures (a), (b), (c), (f), and (g), were incubated at 37 C. The potato glucose agar plates were incubated for 5 days; the "SF" broth cultures and the carrot liver agar shake tubes were incubated for 3 days; and the remaining cultures, procedures (a), (b), (c), and (g), were incubated for 2 days.

Colony counts were made of suitable dilutions of the agar plates and agar shake tubes, with the aid of a Quebec colony counter. The most probable numbers of enterococci as shown by "SF" broth cultures were calculated according to the M. P. N. table in *Standard Methods for the Examination of Water and Sewage* (1946). Since it was impossible to obtain enough intestinal contents from young chicks for dry weight determination, all counts are expressed on a wet weight basis.

Statistical analysis.[3] An analysis of variance was made of the counts obtained

in each cultural procedure except the anaerobic agar plates; the latter counts were so similar to the aerobic agar plate counts that there seemed to be no need for a separate statistical analysis. All statistics were computed with the logarithms of the counts; the mantissa was carried out to two significant numbers. The logs of the counts from chicks up to 141 days of age were included so as to make use of counts obtained from two separate chickens on each day. The eosin methylene blue agar plate counts and enterococcus dilution counts obtained from duodenal contents were omitted from the statistical analysis because in many cases the numbers were so low that no precise counts were obtained. This made the comparison of the counts secured on the contents in the remaining levels of the tract more accurate. F values were determined for variation among levels,
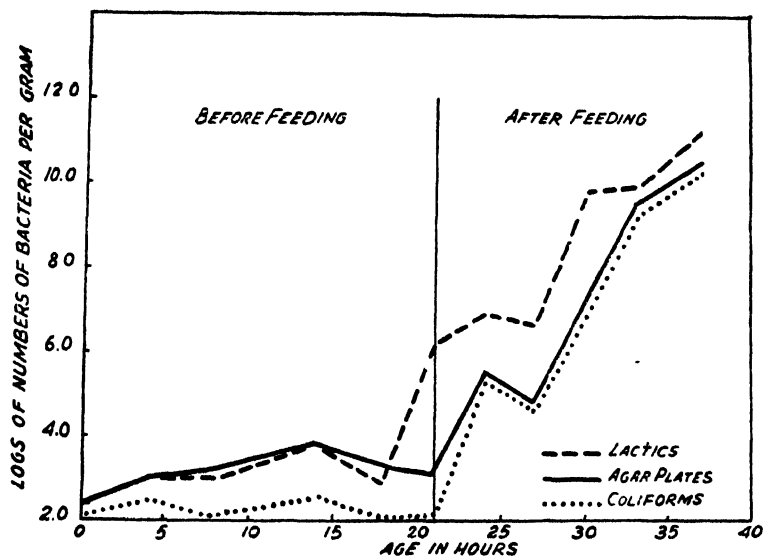
Figure 1. The cecal microflora of young chicks at various ages.

for variation among days, and for the interaction between levels and days. Least significant differences (LSD) were calculated for the F values that were significant.

RESULTS

The cecal microflora of chicks from the time of hatching until 37 hours of age. In order to follow the establishment of each group of bacteria in the cecal contents of chicks that had just hatched, samples were obtained at frequent intervals after hatching until the chicks were first given food, and for 16 hours after that time. Only three counts were determined: aerobic agar plate counts; coliform (EMB) plate counts; and carrot liver agar shake tube counts. The results of this, study are summarized in figure 1. At each sampling period, three chicks were killed and the cecal contents pooled and mixed. One sample was taken from this mixture for analysis. The 0-hour age interval was used to designate a sample taken from the mixture of the cecal contents of three chicks that were still in the shell.

No coliforms were detected in the cecal contents during the 21 hours that the chicks were not fed. The agar plate counts showed low numbers of organisms (between 312 to 4,500 per gram) during the first 21 hours. The carrot liver agar shake tube counts paralleled the agar plate counts during the first 18 hours, but then went up to 1.5 million per gram at 21 hours, although the chicks had not yet consumed any food. After the chicks were fed, the counts obtained with all three cultural procedures increased up to the 37-hour sampling period—16 hours after food had been provided. At this time the three counts were of the order of magnitude characteristic of mature chickens. It should be pointed out that the carrot liver agar shake tubes showed gas formation whenever growth appeared. In older chicks, gas was evident only in the lower dilutions, and not in the higher dilutions. This would indicate that the non-gas-producing bacteria, probably lactic acid bacteria, were present in older chicks in higher numbers than the gas producers. In the very young chicks, however, the gas-producing bacteria were present in greater numbers than the lactic acid bacteria. The kinds of gas producers present were not identified, but it is certain that they were not coliforms.

The Intestinal Microflora of Chickens from Hatching to Maturity

After the initial establishment of the cecal microflora had been observed, samples were taken from four levels of the intestinal tracts of chickens from the age of 1 day through 200 days. Two chicks were sampled at each age interval and the arithmetic means of the counts on each chick obtained with each cultural procedure were used in plotting the graphs. Throughout this work, no yeasts or molds were detected in plates inoculated with dilutions of intestinal contents as low as 1:50. Anaerobic agar plate counts were very similar to the aerobic agar plate counts and therefore only the aerobic agar plate counts are plotted on the graphs. The data for four counts are plotted for each level of the intestinal tract studied and are shown in figures 2 to 5.

The cecal pouches. The numbers of all groups of bacteria were highest in the cecal pouches (figure 2). It can be seen that the numbers of all groups of bacteria showed an initial decline during the first 4 weeks. The numbers of enterococci decreased gradually as the chicks became older, but the other counts showed no clear trends in numbers at any other time. It is interesting to note that the coliform counts seem to follow the agar plate counts quite closely, suggesting that coliforms make up most of that part of the population determined by aerobic agar plate counts. The isolation and identification of organisms growing in the agar plates verified this observation. It is also interesting to observe that the lactic counts were considerably higher than the agar plate counts. The enterococcus counts were lower than the coliform counts at all times.

The colon. The numbers of all groups of bacteria were lower in the colon (figure 3) than in the cecal pouches. None of the groups showed a discernible trend in numbers as the chickens grew older. The coliforms in the colon contents did not appear to constitute so large a portion of the population determined by the agar plate counts as in the cecal pouches. The agar plate counts were much closer to the lactic counts than in the cecal pouches. In the colon, the coliform
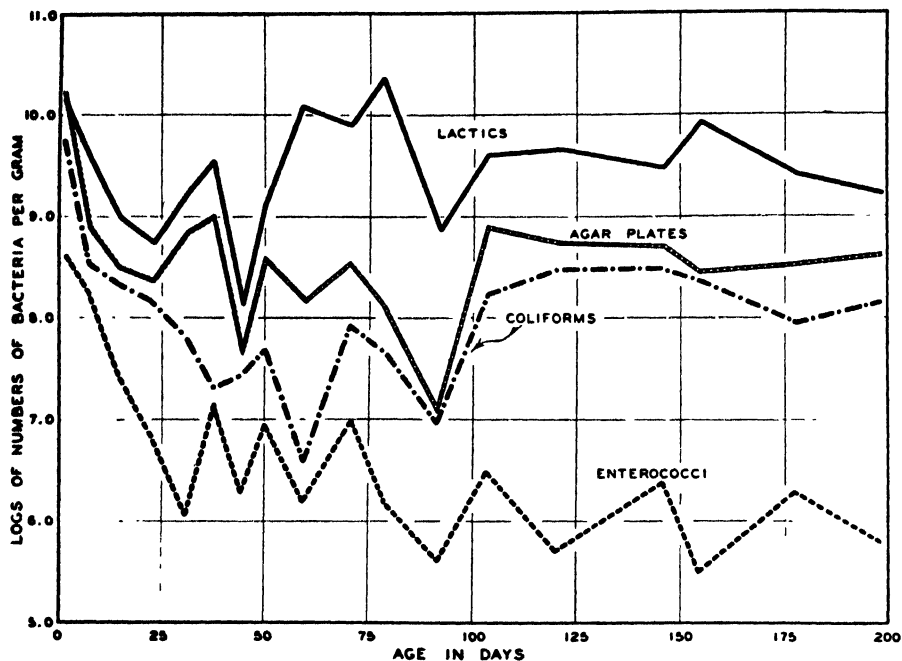
Figure 2. Flora of the cecal pouches.
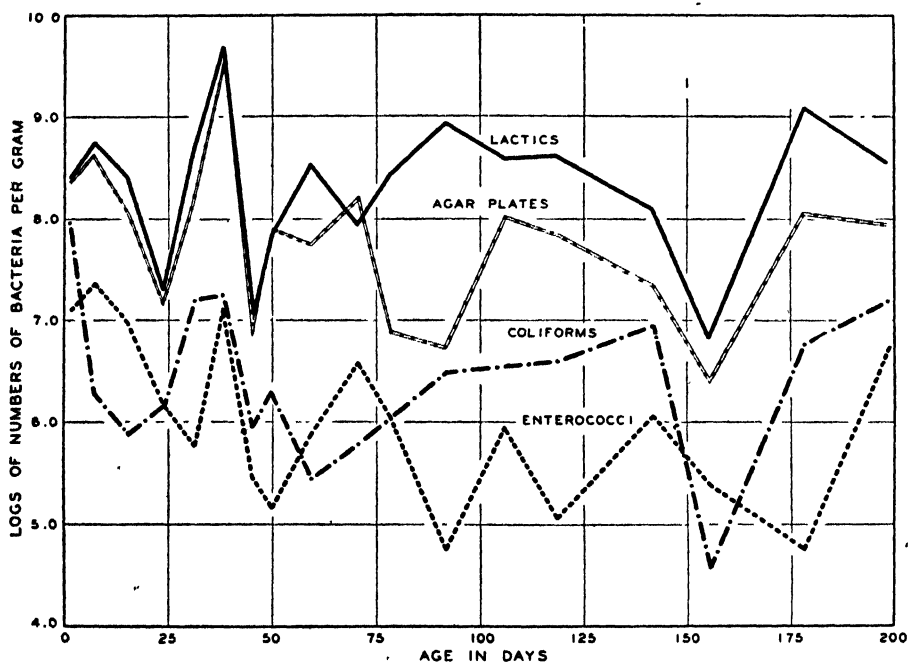
Figure 3. Flora of the colon.
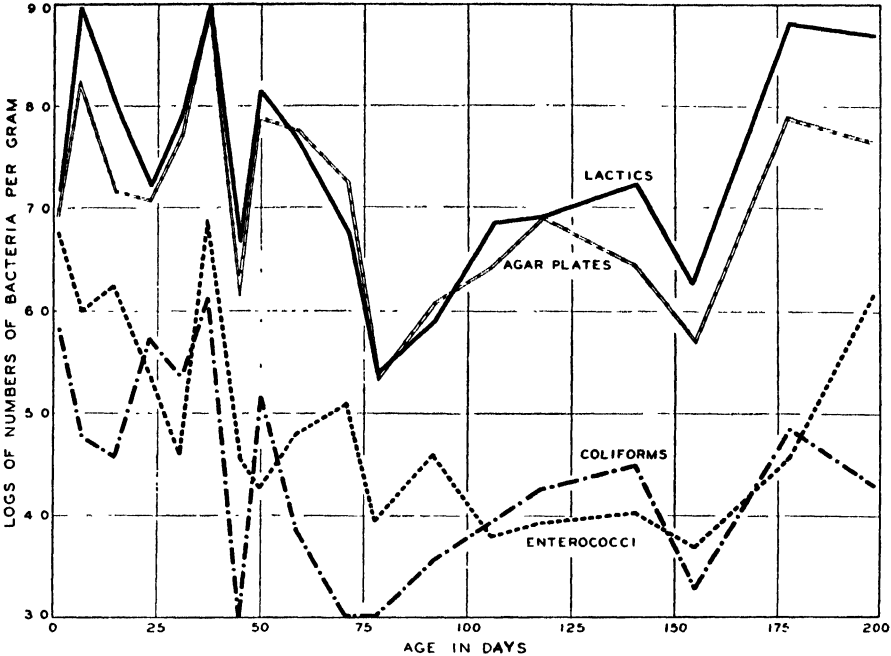
Figure 4. Flora of the ileum.
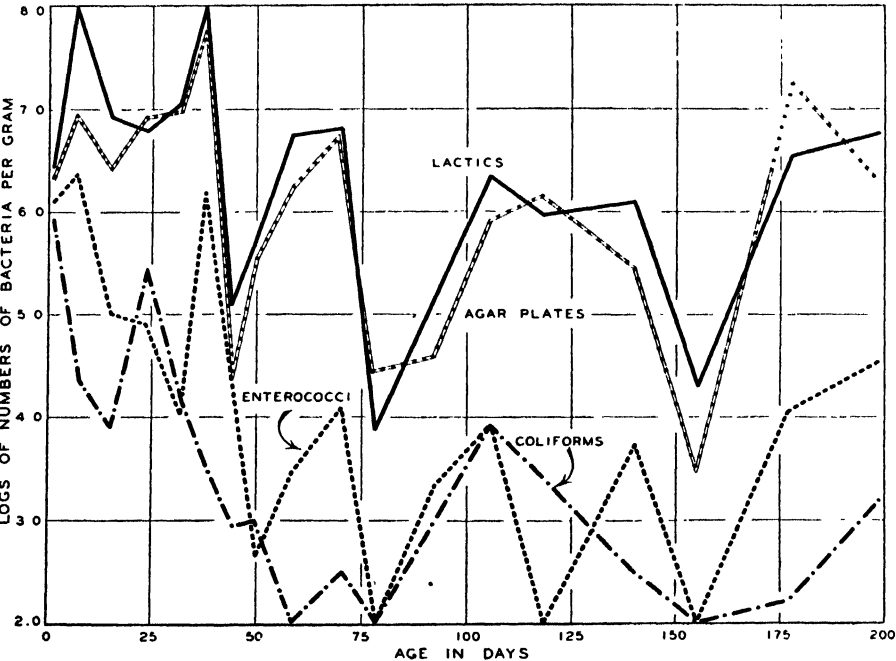
Figure 5. Flora of the duodenum.

and enterococcus counts were more similar than in the cecal pouches, where the numbers of coliforms were considerably higher than the numbers of enterococci.

The ileum. In the ileum (figure 4) the numbers of all groups of bacteria were lower than in the cecal pouches. It can be seen that the numbers of all groups of bacteria decreased gradually until about 11 weeks. The counts then leveled off, except for two high readings at 178 and 199 days. Coliforms made up a very small portion of that part of the population determined by agar plate counts; the agar plate counts and lactic counts were practically identical. The numbers of enterococci and coliforms were very similar.

The duodenum. As can be seen in figure 5, the numbers of all groups of bacteria were lowest in the duodenal contents. The numbers of all groups of bacteria decreased gradually as the chicks grew older until about 11 weeks, and then leveled off, except for two high readings at 178 and 199 days. As in the ileum, coliforms made up a very small portion of that part of the population determined by agar plate counts, whereas the lactic counts and agar plate counts were very similar.

Aerobic and anaerobic agar plate spore counts were made of the contents of the gizzard, duodenum, ileum, cecal pouches, and colon. All counts were of the same order of magnitude at all levels of the tract (1 to 6 $\times$ 10^5 bacteria per gram, wet weight). This seems to indicate that the spores were transient forms which passed through the intestinal tract of the chicken.

It can readily be seen in figures 2 to 5 how jagged the lines for each count appear on the graphs. The day-to-day variation encountered was very great. At each sampling period two birds were sacrificed and the difference in counts between birds was sometimes as great as 1,000-fold. This great variation makes it difficult to discover any trends or to evaluate the results secured. For this reason a statistical analysis of the results was believed necessary.

Variation among levels of the tract. The results of analyses of variance for the counts obtained with various media are summarized in table 1. It is interesting that in spite of the great variation in counts within each level of the tract, the differences among all counts at different levels of the intestinal tract were highly significant (significant at the 1 per cent level of significance). Least significant differences were calculated for the variation among levels. Since the carrot liver agar shake tube counts showed interaction between levels and days, a least significant difference for levels could not be determined for this count. Variation of the counts of the other three cultural procedures was not significantly different among levels on different days, so that least significant differences for levels could be calculated. It was found that the arithmetic means of the aerobic agar plate counts (expressed as logarithms) of the contents of the four levels of the intestinal tract were significantly different from one another. A difference of log 0.28 was needed to detect a significant change at the 5 per cent level of significance. Similarly the arithmetic means of the coliform plate counts (expressed as logarithms) of the contents of the three levels of the intestinal tract were significantly different from one another. A difference of log 0.40 was needed to detect a significant change at the 5 per cent level of significance. The arithmetic means of the entero-

TABLE 1

Analysis of variance of the counts from different levels of the intestinal tract of chickens at various ages

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F VALUE
1. Carrot liver agar shake tube counts				
Among days......	14	46.7464	3.3390	1.6152 N. S.
Between chickens within days.....	15	31.0095	2 0673	
Among levels	3	159.6993	53.2331	115.2731*
Days × levels	42	43.1833	1.0282	2.2265*
Levels × chickens within days..	45	20.7808	0.4618	
Total.....	119	301.4193		
2. Aerobic agar plate counts				
Among days...	14	61.9472	4.4248	4.0906*
Between chickens within days.....	15	16.2259	1.0817	
Among levels	3	104.5522	34.8507	124.6448*
Days × levels..	42	19.2551	0.4585	1.6398 N. S.
Levels × chickens within days....	45	12.5832	0.2796	
Total................	119	214.5636		
3. Coliform ("EMB" medium) plate counts				
Among days.........	14	41.2963	2.9497	1.8614 N. S.
Between chickens within days..... .	15	23.7703	1.5847	
Among levels......................	2	196.2331	98.1166	173.0146*
Days × levels..	28	23.7640	0.8487	1.4966 N. S.
Levels × chickens within days..	30	17.0129	0.5671	
Total.........................	89	302.0766		
4. Enterococcus ("SF" medium) dilution counts				
Among days.....	14	58.1692	4.1549	5.6216*
Between chickens within days......	15	11.0872	0.7391	
Among levels	2	46.4555	23.2278	90.8045*
Days × levels	28	9.5610	0.3415	1.3350 N. S.
Levels × chickens within days.....	30	7.6737	0.2558	
Total.........................	89	132.9466		

N. S.—not significant.
* Significant at the 0.01 level of significance.

coccus dilution counts (expressed as logarithms) of the contents of the three
levels of the intestinal tract were also significantly different from one another.

Here a significant difference of log 0.27 was needed to detect a significant change at the 5 per cent level of significance.

Variation among days. Comparing the counts obtained on different days, it was found that there was a significant difference among the counts obtained on different days with the aerobic agar plates and the enterococcus dilution tubes (both significant at the 1 per cent level of significance). The significant difference among days for the enterococcus counts is probably due to the decrease in these counts with age, observed in the contents of the cecal pouches and ileum (figures 2 and 4). The significant difference among days for the aerobic agar plate counts does not seem to be reflected in any over-all trends. Least significant differences
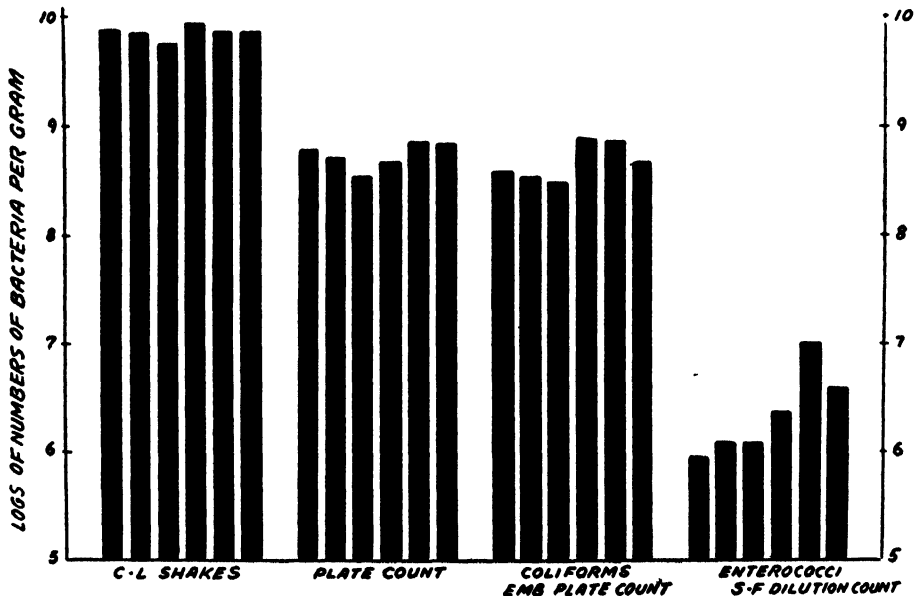
Figure 6. Samples of cecal contents.

were calculated for the variation among days in the aerobic agar plate counts and the enterococcus dilution counts. A difference of log 1.11 was required to detect a significant difference in the aerobic agar plate counts, and a difference of log 1.06 was required to detect a significant difference in the enterococcus dilution counts.

In order to check on the accuracy of the enumerative techniques employed, a cecal sample was taken and divided into six equal portions. Three replicates of this mixed sample were mixed with glass beads, and three were mixed in a War-, ing blender. The same cultural procedures as described earlier were used to determine the numbers of bacteria in the cecal sample. The results are summarized in figure 6. It will be seen that there is good agreement between the carrot liver agar shake tube counts, and between the aerobic agar plate counts, and fairly good agreement between the enterococcus dilution counts made on replicates of cecal samples. In each case the first three counts (from left to right) were

obtained from samples mixed with glass beads, and the last three counts were obtained from samples mixed in a Waring blender for one minute. The Waring blender counts were slightly higher than counts obtained from samples mixed with glass beads. This was especially true in the enterococcus counts, perhaps because of the breaking up of chains.

Replicate samples of colon contents were tested in the same manner used for cecal samples with essentially similar results. It appears, then, that the variations encountered between different birds are not due to inherent inaccuracies in the enumerative technique; apparently there is that much variation between the animals. When one considers the number of factors influencing the mixed flora of the intestinal tract, it is reasonable to expect great variation, for at any time when samples might be taken it is clear that variation in any one of these factors —such as the amount of contents in the tract—might profoundly influence the numbers of bacteria that will be determined by plating or by dilution count procedures.

The intestinal microflora of chickens that had been reared on range was found to be similar to that of chickens raised in battery cages. The relationships of the numbers of the different groups of bacteria to one another at each level of the intestinal tract were similar to those obtained with chickens raised in cages. However, eosin methylene blue agar plates from the cecal contents of range chickens showed roughly 10 to 25 per cent of the colonies to be the *Aerobacter aerogenes* type, whereas similar plates from cecal contents of chicks kept in battery cages rarely showed any *A. aerogenes* colonies. The presence of the *Aerobacter* organisms is no doubt due to the availability of plant and soil sources of contamination of the food consumed by chicks on range.

Isolation and identification of cultures from some of the principal groups of bacteria found after the normal flora had become established in the intestine of the chicken provided information of value in arriving at an explanation of the quantitative data.

Coliforms. Two hundred and fifty cultures were isolated and all were identified as typical *Escherichia coli* cultures. Apparently *E. coli* makes up the entire coliform population. Furthermore, the eosin methylene blue agar medium used served as a very good measure of the numbers of coliforms, for higher numbers of coliforms were not found when any other medium was used.

Lactic acid bacteria. It was found that the carrot liver agar medium used in these studies served as a measure of the numbers of lactic acid bacteria that were present in the samples. With samples of the contents of the duodenum, ileum, and cecal pouches, the carrot liver agar shake tube counts represented a good estimate of the numbers of lactobacilli. The lactic counts of the colon samples, however, are higher than the numbers of lactobacilli that are present. Lactobacilli were found to be the most numerous group of bacteria in most areas of the intestinal tract. Since the vitamin requirements of these organisms are known to be quite extensive, it is likely these bacteria play an important role in the vitamin economy of the host. Hence a detailed study of this group of intestinal bacteria was undertaken and will be reported separately.

The "SF" broth was found to be a very selective medium. Only enterococci

were found to grow in this medium and so the counts obtained for enterococci were considered quite reliable. It was found, however, that enterococci appeared in the carrot liver medium in higher numbers than would be indicated from the "SF" dilution counts obtained from the same sample. This was true in samples of the contents of the colon and cecal pouches. Thus, it appears that the "SF" dilution counts for enterococci were lower than the actual numbers of enterococci that were present in the contents of the colon and cecal pouches. One hundred and five cultures of enterococci were isolated from "SF" broth and carrot liver agar tubes. All cultures were identified as *Streptococcus faecalis*. Apparently this organism makes up the entire enterococcus population.

Anaerobes. Re-examination of the anaerobic flora of the normal chicken has confirmed the earlier report of Johansson and Sarles (1948) that the principal obligate anaerobe is *Clostridium perfringens*. Attempts to isolate other anaerobes, particularly putrefactive anaerobes, have not been successful.

DISCUSSION AND SUMMARY

Quantitative studies on the intestinal microflora of normal chickens at various ages have revealed that newly hatched chicks harbor very few microorganisms in their intestinal tracts. This remains true for the 21-hour period following hatching, during which the chicks were not fed, except for the carrot liver agar shake tube counts, which showed a significant increase in numbers at the twenty-first hour after hatching. Gas-producing microorganisms were present at the twenty-first hour in high numbers (1,500,000 per gram), but these microorganisms were not isolated in pure culture. These organisms were not coliforms, since no coliforms were detected on the eosin methylene blue agar plates inoculated with the same samples as were the carrot liver agar shake tubes. After the chicks were given food and water the numbers of all groups of bacteria increased very rapidly until 16 hours after the chicks were fed, when the numbers reached a peak.

Qualitative studies of the establishment of the microflora would serve to complement the quantitative results obtained. Of special interest would be the identification of the organisms that become established in the cecal pouches of chicks before they consume any food. It would also be valuable to determine the conditions which permit the lactobacilli to establish themselves as the most numerous species of bacteria in the contents of the intestinal tract and at what time they become established.

The results show that the "normal numbers" of bacteria became established in the contents of the duodenum, ileum, cecal pouches, and colon after the chicks had been given food and water for only 16 hours (within 40 hours after hatching). The numbers of bacteria of all groups studied were found to be highest in the contents of the cecal pouches and progressively lower in the contents of the colon, ileum, and duodenum.

A statistical analysis of the quantitative results showed that the counts obtained with each cultural procedure were significantly different among the different levels of the intestinal tract. It was found that at each level of the intestinal tract the arithmetic means of the aerobic agar plate counts, the coliform plate

counts, and the enterococcus dilution counts were significantly different from the corresponding arithmetic means obtained with each cultural procedure at any other level of the intestinal tract.

In comparing the differences in counts obtained with each cultural procedure on different days without regard to levels of the intestinal tract, it was found that only the enterococcus dilution counts and the aerobic agar plate counts showed significant differences among days. The differences in the enterococcus dilution counts among days are probably a reflection of the decrease in counts observed with this cultural procedure in the contents of the cecal pouches and ileum.

To determine whether the enumerative techniques would give consistent results, experiments were conducted in which one sample was divided into several replicates. In these cases, the replicate counts obtained with all cultural procedures showed very good agreement. This indicates that the techniques employed to obtain counts on the intestinal flora of each of two chickens on any one day contributed very little variation to the results obtained.

The relationships of some of the groups of bacteria to one another were characteristic of each level of the intestinal tract. Larger numbers of coliforms in proportion to the total agar plate counts were found in cecal pouch contents than in colon contents. In the ileum and duodenum it was found that the coliforms made up a very small portion of that part of the population determined by the agar plate counts. The agar plate counts were much lower than the lactic counts made on the cecal pouch contents. However, in the colon, ileum, and duodenum, the lactic counts and agar plate counts were very similar. In addition, it was found that the enterococcus and coliform counts were very similar in the duodenum, ileum, and colon. In the cecal pouches, the numbers of enterococci were lower than the numbers of coliforms. The anaerobic agar plate counts paralleled the aerobic agar plate counts so closely in all cases that no significant differences could be observed between the two counts.

Unidentified species of lactobacilli appear to be the most numerous group of bacteria in all levels of the intestinal tract of the chicken, with the exception of the colon.

Escherichia coli was found to be the predominant coliform, but was of numerical significance only in the contents of the colon and cecal pouches. *Streptococcus faecalis* was present in large numbers only in the cecal pouches and colon.

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NOTES

A POOLED PARACOLOBACTRUM INTERMEDIUM ANTISERUM FOR SCREENING IN ENTERIC BACTERIOLOGY[1]

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Recent investigations of the antigenic relationships of all *Paracolobactrum* cultures isolated in the Florida State Board of Health, Jacksonville, laboratory during 1946 and 1947 (Stuart, Galton, and McGann: J. Bact., **46**, 411, 1948) revealed that 40 per cent of these cultures were *Paracolobactrum intermedium*. It was also found that many of the *P. intermedium* cultures belonged to the Bethesda group described by Edwards, West, and Bruner (J. Bact., **55**, 711, 1948). Because of the biochemical similarity of these organisms to *Salmonella*, a great deal of time and many media and frequently *Salmonella* antiserums are necessary for differentiation.

In an effort to obtain a rapid method of screening these cultures in a diagnostic laboratory handling large volumes of routine enteric cultures, a pooled *P. intermedium* antiserum was prepared. This antiserum consisted of a pool of 6 of the 9 basic antiserums used in the original study of the 765 *P. intermedium* cultures (Stuart, Galton, and McGann, 1948). Those selected for the pool were 5883, 7328, 14011, 20157, 20158, and 20565. They were pooled in amounts of 2.5 ml each.

All cultures giving a suspicious *Salmonella* reaction on Kligler's iron agar slants (KIA, 1 per cent sucrose added) were subsequently tested for urea breakdown and indole production. If both tests were negative the cultures were slide-tested in a 1:5 dilution of the pooled *Paracolobactrum* antiserum.

After approximately 500 KIA slants that showed a suspicious *Salmonella* reaction with this pooled *P. intermedium* antiserum were checked, no positive reactions were obtained with the cultures that failed to produce H₂S. This was not surprising as, with the exception of an occasional variant, all of the *P. intermedium* cultures that have been studied by various investigators have produced H₂S. It was also noted that the suspicious KIA slants that produced H₂S were rarely urea- or indole-positive. Therefore, the procedure shown in diagram 1 was used routinely. This method has reduced by more than 50 per cent the number of rapid urea and indole tests performed on suspicious KIA slants forming H₂S.

During the past four months the pooled *P. intermedium* antiserum has been in use routinely in the examination of 3,718 fecal cultures in the Jacksonville laboratory. From 263 specimens, cultures that showed acid and gas in KIA and

[1] Dr. P. R. Edwards, who very kindly reviewed this paper, states that they have used similar pooled serums for screening and find them helpful.

formed H$_2$S were isolated. Of these, 149 showed positive slide tests with the pooled *P. intermedium* antiserum; 39 proved to be *Salmonella*; 33 were urea-positive; 6 were indole-positive; and 36 failed to agglutinate with either the polyvalent *Salmonella* or pooled *P. intermedium* antiserums, were urea- and indole-negative, and required further cultural and serological study.

These results clearly indicate the value of a pooled *Paracolobactrum intermedium* antiserum as an additional screen aid in the isolation and identification of *Salmonella* from fecal specimens.

Diagram 1

Outline of Procedure for the Identification of *Salmonella*

Kligler's iron agar; AG, H$_2$S + only

Spot with polyvalent
Salmonella antiserum

+   −

Type with specific
Salmonella serums

Spot with pooled
P. intermedium serum

+   −

P. intermedium

Inoculate media for biochemical
confirmation and serological tests

Discard if + in any one medium
{ 24-hour urea medium
  " " tryptone broth
lactose broth
sucrose broth

Use for serological study
{ infusion broth
  " agar slant
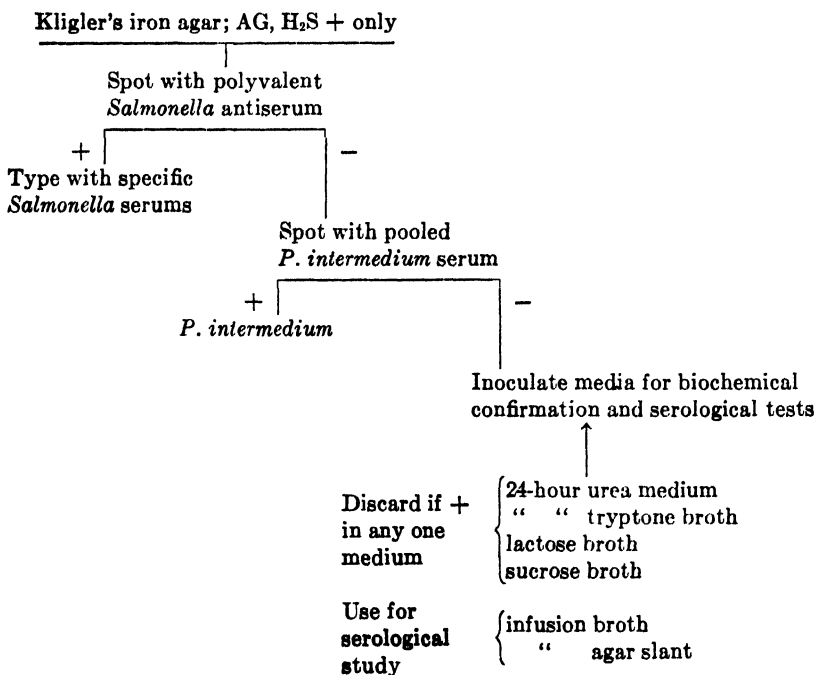
Note: Since this report was written a satisfactory "polyvalent" *P. intermedium* antiserum has been prepared by injection of a pooled antigen consisting of the 9 basic *P. intermedium* strains into one rabbit.

NO ANTISTREPTOMYCIN ACTIVITY SHOWN BY INOSITOL PHOSPHOLIPIDS

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Lipositol, an inositol-containing phospholipid material obtained from soy beans (Woolley: J. Biol. Chem., **147,** 581, 1943) was reported to show antistreptomycin activity, with 0.2 μg per ml nullifying the antibacterial action of 60 μg per ml of streptomycin (Rhymer *et al.*: J. Biol. Chem., **169,** 457, 1947). Similar materials showing antistreptomycin activity were obtained from brain and plant sources (Rhymer and Wallace: J. Bact., **54,** 521, 1947). Since the streptomycin molecule contains *meso*-inositol, the former observation suggested a metabolite-antimetabolite relationship. Confirmation of this observation has not been noted.

In this laboratory three phospholipid materials were tested for antistreptomycin activity: (1) soybean monophosphoinositide (containing inositol monophosphate, glycerol, and carbohydrate) (Folch: Federation Proc., **6,** 252, 1947), (2) cattle brain cephalin diphosphoinositide (containing inositol *meta*-diphosphate, glycerol, and fatty acids) (Folch: J. Biol. Chem., **177,** 505, 1949), and (3) serine phosphatide (containing no inositol), all of which were kindly provided by Dr. J. Folch. These materials were tested by incorporating them into the liquid synthetic medium[2] used in a series of onefold serial dilution streptomycin sensitivity assays against a strain of *Escherichia coli* (841). Growth of this organism was completely inhibited by 6 μg per ml of streptomycin.

In these experiments there was no evidence that either inositol-containing phospholipids, obtained from soybeans and brain, or serine phosphatide in concentrations up to 200 μg per ml interfered with the antibacterial action of 6 μg per ml of streptomycin.

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[1] Fellow in the Medical Sciences, National Research Council.

[2] $(NH)_2SO_4$, 2.5 g; KH_2PO_4, 0.6 g; K_2HPO_4, 3.4 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; glucose, 10 g; water, 1,000 ml; pH 7.1.

A SIMPLIFIED METHOD FOR SEALING PETRI DISHES[1]

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Considerable difficulty has been experienced in the use of petri dishes for the isolation and cultivation of slow-growing organisms such as *Mycobacterium tuberculosis*. To prevent the drying of the medium Petroff (Proc. Soc. Exptl. Biol. Med., **24,** 632, 1926) recommended the use of broad rubber bands to seal the dishes; others have used adhesive tape, cellulose tape, or paraffin. These methods are cumbersome and frequently ineffective.

In the course of some of our studies necessitating the prolonged incubation of bacterial cultures we have found the use of polyethylene transparent plastic envelopes an effective, rapid, and simple method. This product is manufactured by various companies as a flat tubing of different diameters and thicknesses.[2] It is cut to the desired length, the inoculated petri dish is inserted into the tube, and both ends are then sealed by means of a small electric heater[3] or by means of an electric flatiron, when the ends to be heated are protected with a piece of cellophane. The colonies may be studied through the thin envelope, although if desired the envelope may be opened, the culture examined, and the dish resealed in the envelope. For this reason, we recommend that the envelope be an inch or more longer than the dish.

[2] Obtained from the Plax Corporation, Hartford, Conn.

[3] Dobeckmun Company, Cleveland, Ohio.

THE GROWTH OF MYCOBACTERIUM TUBERCULOSIS AS A FUNCTION OF ITS NUTRIENTS

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Dubos and Davis (1946) and Davis and Dubos (1947) have shown that *Mycobacterium tuberculosis* gives uniform growth throughout a liquid nutrient medium in the presence of a wetting agent, sorbitan monooleate, and albumin. It has become possible, therefore, to use turbidimetry for the quantitative study of the growth of this bacillus.

The experiments to be reported here show that the growth of *Mycobacterium tuberculosis* in the Dubos medium (Dubos and Middlebrook, 1947) can be followed quantitatively by turbidimetry. With the procedures described it has been possible to analyze the role of glucose as a carbon source and of ammonium chloride as a nitrogen source for the growth of tubercle bacilli. It has been found that under certain conditions, i.e., with glucose available but with ammonium chloride limiting, autolysis of these bacteria occurs, and also that, when the rapidly growing bacteria are deprived of ammonium chloride, the turbidity of the culture will continue to increase for a time in the absence of an external supply of nitrogen. The human tubercle bacillus, strain H37Rv,[1] was used in all these experiments.

EXPERIMENTAL METHODS AND RESULTS

Method for measurement of growth. The validity of turbidimetric procedures for a quantitative measurement of the growth of *Mycobacterium tuberculosis* in Dubos medium was tested by the following experiments:

(1) Various dilutions of a suspension of tubercle bacilli, grown for 10 to 12 days in Dubos medium, were prepared and introduced into culture tubes of the screw-cap type; their optical densities were measured with the Coleman-Universal spectrophotometer. Readings were made in blue (λ 480 mμ) and red (λ 650 mμ) light. The results recorded in figure 1 show that the relationship between the dilution of the suspension and its optical density was linear for readings in blue light to an optical density of 0.500 and for readings in red light to an optical density of 0.600. Although the absorption was greater in blue light than in red, in the latter case the linearity of the optical density curve extended over a larger range. Therefore, red light was used for most of the readings.

(2) The optical density and the dry weight of a culture grown in Dubos medium were compared at different stages of growth in the following way: 24 Roux bottles, each containing 200 ml of medium, were inoculated with 2 ml of a

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[1] Received through the courtesy of Dr. R. Dubos of the Rockefeller Institute.

10-day-old culture. After 4, 6, 8, 10, and 12 days, 4 bottles were removed, their contents mixed, and the total volume and the optical density measured. Then formalin was added to a concentration of 1 per cent in the culture, and after several days the bacteria were collected by centrifugation and dried to constant weight in an oven at 80 C.
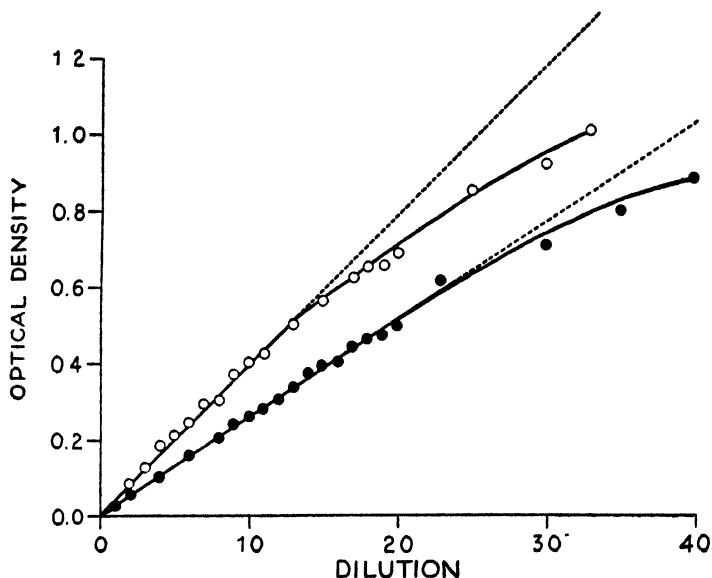
Figure 1. Optical density measurements as a function of the dilution of a suspension of tubercle bacilli. Optical density λ 480 mμ, O—O; optical density λ 650 mμ, ●—●.

TABLE 1

The relationship between the optical density and the dry weight of a culture of Mycobacterium tuberculosis during growth

AGE OF CULTURE IN DAYS	OPTICAL DENSITY	DRY WEIGHT OF BACTERIA MG/L	DRY WEIGHT OF BACTERIA MG/L PER UNIT OF O.D.
4	0.264	120.1	455 } average 455
6	0.372	166.7	448
8	0.606	279.3	461
10	0.959	485	506
12	0.097	578.4	527

The results given in table 1 show that the proportion of the dry weight of the formalinized bacteria to the optical density, during the growth, remained fairly constant. The ratio of the dry weight of bacteria in mg per liter to optical density for all measurements between the fourth to the eighth day was 455 mg per liter ± 5 per cent per unit of optical density. For later dates (tenth and twelfth day) the ratio increased to 500 and 520. It follows from this experiment that it is possible to calculate from the optical density reading the dry weight of bacterial substance grown in Dubos medium, with an accuracy of about 10 per cent.

Influence of the dispersing agent. Polyoxyethylene sorbitan monooleate ("tween 80"), the dispersing agent of the culture medium, contains in its commercial form a certain amount of free oleic acid, which is highly toxic for the tubercle bacillus. It is rendered nontoxic by admixture with serum albumin, which binds and neutralizes the free oleic acid (Davis and Dubos, 1947). Detoxified sorbitan monooleate may affect the growth of the tubercle bacillus in a nutrient medium not only by changing the optical properties of the culture, but also by acting as an additional nutrient and thus increasing the total amount of growth; it may also accelerate the growth rate in the presence of optimal amounts of other nutrients. The latter possibility was tested in the following experiment:

Various quantities of "tween" were added to different batches of medium containing optimal amounts of nutrient (NH_4Cl 0.5 mg per ml, glucose 10 mg per ml, albumin 4 mg per ml, and a salt mixture). Each batch was inoculated with 0.01 of its volume of a 10-day old culture. Then samples were distributed in culture tubes and the optical density was measured daily.

It was observed that a concentration of sorbitan monooleate at or above 0.01 per cent gave dispersed growth. Below that concentration, growth was granular. Growth was more rapid with 0.025 per cent sorbitan monooleate than with 0.01 per cent, but higher concentrations did not produce further increase in the growth rate. The growth rate of the bacteria without sorbitan monooleate could not be measured by turbidimetry because the bacteria were not dispersed. However, visual inspection of the centrifuged bacteria showed that the growth without sorbitan monooleate was considerably less than with it. It appeared from this experiment that sorbitan monooleate acted not only as a dispersing agent, but also as an accelerator for the growth of *Mycobacterium tuberculosis* as previously demonstrated by Dubos (1947). Since the maximal accelerating effect was reached at 0.02 per cent, this amount was used in subsequent experiments.

Influence of albumin on the growth of Mycobacterium tuberculosis. Albumin was studied with respect to its nutritive value as a carbon and as a nitrogen source. When used as the only carbon source, with ammonium chloride as the nitrogen source, a concentration of 0.1 per cent albumin permitted no measurable growth. With 0.2 per cent albumin, growth reached an optical density of 0.02, and with 0.4 per cent albumin, an optical density of 0.07. The nutritive value of albumin as a nitrogen source was studied in the presence of 10 mg per ml glucose. Ferric chloride (0.0005 per cent) was used instead of ferric ammonium citrate. Each concentration of albumin was tested in four parallel experiments. The maximal optical density after 10 days of growth varied from 0.03 to 0.07 with 0.1 per cent albumin, from 0.04 to 0.12 with 0.2 per cent albumin, and from 0.05 to 0.15 with 0.4 per cent albumin. These results showed that the albumin possessed nutrient properties both as a carbon and as a nitrogen source, but the amount of growth was very small when a concentration of 0.1 per cent was used. This concentration was chosen for subsequent experiments.

The effect of various concentrations of albumin on the growth rate of *Mycobacterium tuberculosis*, when all the other nutrients were supplied in optimal amounts, was also studied. It was observed that a variation in the albumin concentration

from 0.1 to 0.4 per cent did not exert a marked influence on the rate of growth, but, in the absence of albumin, growth did not occur. It can therefore be concluded, in agreement with Dubos and Davis (1946) and Davis and Dubos (1947) that the principal function of albumin, under the conditions obtaining in these experiments, was that of a detoxifying agent.

Culture medium and experimental procedure. The salt mixture had the following composition: KH_2PO_4, 1 gm; $Na_2HPO_4 \cdot 12H_2O$, 6.3 gm; $MgSO_4 \cdot 7H_2O$, 0.01 g; ferric ammonium citrate, 0.05 g; $CaCl_2$, 0.0005 g; and distilled water, 1 liter. In some of the experiments 0.005 g of ferric chloride were used in place of the ferric ammonium citrate.[2]

One hundred to 200 ml of the salt mixture containing 0.02 per cent sorbitan monooleate were put in a Roux bottle. After sterilization, albumin[3] was added to a final concentration of 0.1 per cent. As inoculum, a 10-day-old culture, grown in Dubos medium, was used. This culture was washed twice with the basal medium by centrifugation, resuspended, and introduced into the culture medium in such an amount that a 1:100 dilution was obtained. The Roux bottle was incubated at 37 C in a horizontal position and shaken once daily.

In order to reduce the nutrient properties of this basal medium, the bacilli were permitted to grow in this medium until growth almost stopped.[4] This happened in 4 days. Then a solution of ammonium chloride was added to the culture in order to obtain the desired concentration of the nitrogen source and the culture was distributed with a pipette into 20-by-125-mm culture tubes of the screw-cap type.[5] These tubes were previously selected so that the variations of their optical density did not exceed 1 per cent. Each tube was filled with 5.4 ml of the culture. Finally 0.6 ml of a concentrated solution of the carbon source was added in order to obtain the desired concentration. To one tube distilled water was added instead of the carbon source. This tube was the control for the nutrient properties of the basal medium.

Surface area of the culture medium. Growth in vertical culture tubes containing 6 ml of medium in a 30-mm-high column of liquid was compared with growth in slanted tubes ($\ll 10°$) containing a shallow layer (0.1 to 0.5 mm high) of the same volume of medium. All tubes were shaken once a day briefly before the reading of the optical density. The experiment showed that growth was more rapid in the slanted cultures, suggesting that aeration was a significant factor (figure 2).

Influence of the shaking on growth. Mycobacterium tuberculosis was cultured in two sets of several 50-ml Erlenmeyer flasks containing 20 ml of medium in a 15-mm layer. One set was left immobile; the other was continuously shaken on a shaking apparatus (140 rpm at an amplitude of ¾ inches). The temperature in the flasks kept on the shaking apparatus was identical to the temperature in

[2] Citrate is not a carbon source for *Mycobacterium tuberculosis* (Schaefer, 1948).

[3] A 5 per cent stock solution in saline, neutralized with NaOH, filtered through a Seitz filter, and heated ½ hour at 56 C in a water bath, was used.

[4] Total exhaustion of the basal medium is not obtained by this method since sorbitan monooleate is a carbon source (Dubos and Middlebrook, 1947).

[5] Kimbal Glass Company No. 45066.

the incubator room. One flask of each set was removed daily for measurement of the optical density. It was observed that the cultures that were continuously shaken showed visible bacterial clumps, but those that were not shaken showed homogeneous growth. In addition, there was much less growth in the shaken cultures. The procedure adopted for all subsequent experiments was to grow the cultures in tubes kept in racks in a slanted position and agitated briefly once or twice a day. Evaporation of the medium during incubation was minimized by maintaining the humidity in the incubator at 60 to 70 per cent.

Measurement of the generation time and of the growth rate. The generation time is defined as the time necessary for a bacterial mass to double. Since, during the exponential phase, the mass doubles at a constant rate, it is useful to calculate the generation time by the procedure of Monod (1942a) and to represent the
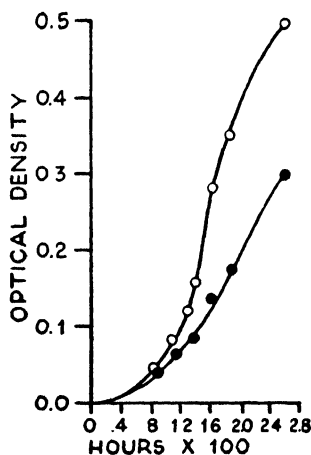
Figure 2. Comparison of the growth in tubes kept in vertical and slanted positions. Medium: NH₄Cl 0.5 mg per ml, glucose 10 mg per ml, sorbitan monooleate, albumin. Slanted position, O—O; vertical position, ●—●.

optical densities by their logarithm to the base 2. The values are plotted as ordinates and the time as abscissae. Under these conditions an increase of 1 in the log₂ of the optical density corresponds to a doubling of the optical density, and the generation time can be read directly on the abscissa. The growth rate is defined as the number of generations per unit of time.

Growth rate as a function of the initial concentration of glucose. As already reported (Schaefer, 1948), the initial concentration of glucose in the medium has a marked influence on the growth rate. This is illustrated by figure 3 and table 2. The growth rate increased with increasing initial glucose concentrations and reached its maximum value at an initial glucose concentration of 10 mg per ml. When the growth rate was plotted as a function of the initial concentration of glucose (figure 4), a curve was obtained which was similar in shape to those found by Monod (1942a) for *Escherichia coli*, *Bacillus subtilis*, and other bacteria. However, the maximum growth rate of these rapidly growing bacteria was

reached at the initial glucose concentration of 0.05 mg per ml, whereas the maximum growth rate of *Mycobacterium tuberculosis* was reached at a concentration of glucose 200 to 1,000 times higher (10 to 50 mg per ml). The possible significance of this difference will be discussed later.

Bacterial yield as a function of the initial concentration of glucose. The total amount of growth in bacterial dry weight was calculated for initial glucose concentrations of 0.100, 0.250, and 0.500 mg per ml. Since growth in the presence of such low concentrations of glucose is very slow, maximal growth was reached only after 2.5 to 3 months. The values of the amount of growth obtained from these various initial glucose concentrations are given in table 3 and represent
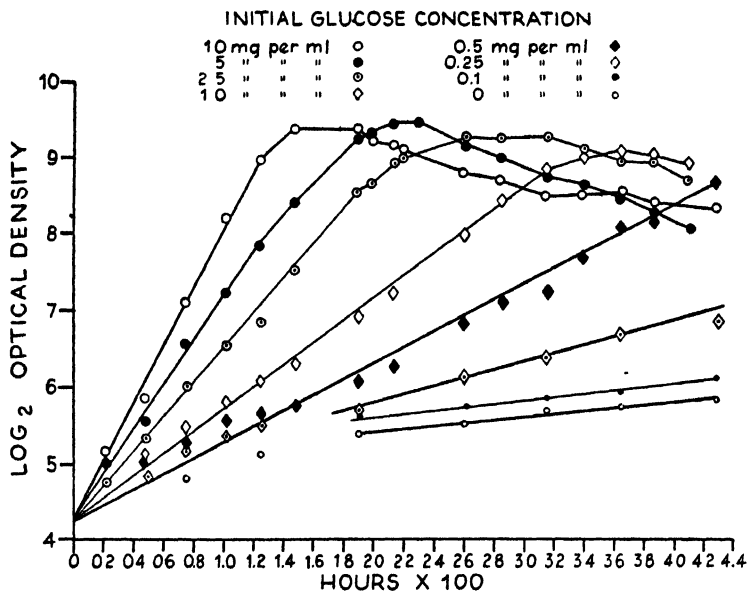
Figure 3. Growth and autolysis of cultures of tubercle bacilli in sorbitan monooleate, albumin medium containing a constant limiting amount of NH₄Cl (0.025 mg per ml) and varying concentrations of glucose.

the average calculated from 11 to 12 experiments.[6] The yield (mg bacterial dry weight per mg glucose) varied from 13 to 25 per cent of the amount of glucose initially added after deducting the growth obtained without glucose.

Growth as a function of ammonium chloride concentration. If glucose is added to the medium in excess and ammonium chloride in small amounts, ammonium chloride becomes the limiting factor for growth. This is shown by the experiment recorded in figure 5 in which NH₄Cl concentrations varying from 0.0025 to 0.1 mg per ml and a constant initial glucose concentration of 10 mg per ml were used. The total amount of growth in mg per liter was calculated from the curves in

[6] Evaporation was found to be approximately 10 per cent at the end of 3 months and was corrected by the addition of distilled water. The dry weight of the bacteria in mg per liter was calculated by multiplying the optical density values by the factor 455.

TABLE 2

The rate of growth and of autolysis of Mycobacterium tuberculosis in the presence of a constant limiting amount of ammonium chloride (0.025 mg per ml) and varying initial concentrations of glucose

INITIAL GLUCOSE CONCENTRATION	GENERATION TIME	TIME REQUIRED FOR 33% DECREASE OF OPTICAL DENSITY	DECREASE OF OPTICAL DENSITY (PER CENT OF MAXIMAL O.D. 276 DAYS LATER)
Experiment 1			
m/gml	*hours*	*hours*	
10	27	70	58–68.5
5	34	60	66
2.5	45	135	55–63
1.0	68	320	45
0.5	96		22
0.25	168		
0.1	460		
Experiment 2			
10	29	115	59
5	37	155	51
2.5	50	160	54
1.0	88	>400	28
0.5	140		10–24
Experiment 3			
10	32	75	69
5	44	100–160	59
2.5	60	160–220	47–59
1.0	74		22–30
0.5	106		
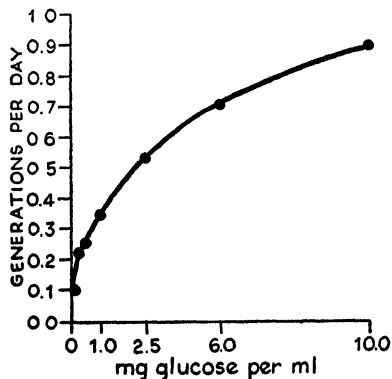
Figure 4. Growth rate of tubercle bacilli as a function of initial glucose concentration.

figure 5 and these values were plotted against the corresponding NH₄Cl concentration to obtain the relationship shown in figure 6. It shows that the total

TABLE 3

The total growth and yield of Mycobacterium tuberculosis cultures on sorbitan monooleate, albumin, salt medium as a function of the glucose concentration

INITIAL GLUCOSE CONCENTRATION IN MG/ML	TOTAL GROWTH IN OPTICAL DENSITY	CORRECTED GROWTH*	TOTAL GROWTH IN MG/L	$\dfrac{\text{TOTAL GROWTH}}{\text{INITIAL GLUCOSE CONCENTRATION}}$ = YIELD
0.500	0.252	0.193	87.8	0.18
0.250	0.198	0.139	63.2	0.25
0.100	0.088	0.029	13.2	0.13
0	0.059			

* Corrected by subtraction of the growth in the absence of glucose.
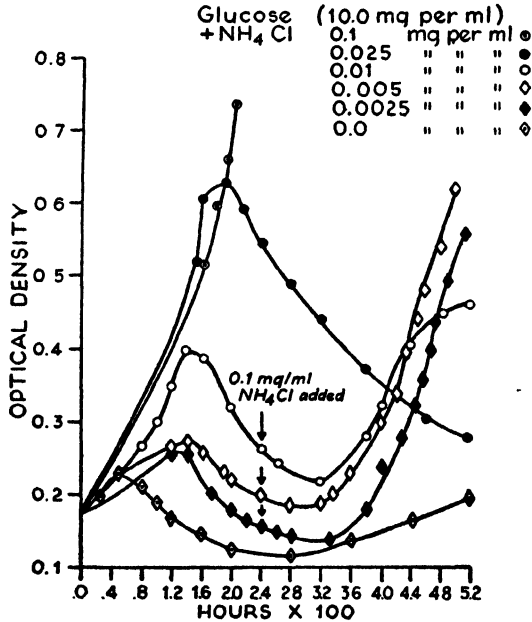
Figure 5. Effect on growth of the addition of ammonium chloride after exhaustion of the nitrogen source in the medium.
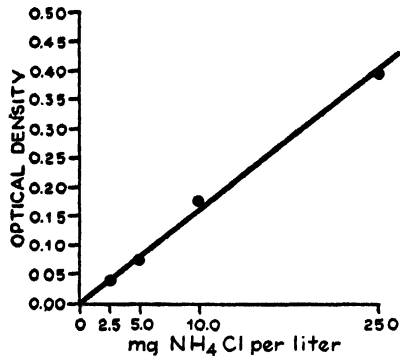
Figure 6. The amount of growth of tubercle bacilli as a function of initial ammonium chloride concentration.

amount of growth was proportional to the initial amounts of ammonium chloride added to the medium.

The influence of various initial concentrations of ammonium chloride (0.001 to 1 mg per ml) on the growth rate of tubercle bacillus, in the presence of a constant initial glucose concentration (10 mg per ml), was also studied. The growth rate was found not to vary with varying ammonium chloride concentraions (table 4) when concentrations of 0.001 mg per ml or higher were added.

The phase of decline. When the supply of NH₄Cl was the limiting factor for growth and when the growth of the culture had increased to the maximum, the optical density remained at this level for a short time and then decreased, at first quite rapidly but later at a slower rate (figures 3 and 5). This decline in the optical density represents autolysis of the bacteria.

TABLE 4

The rate of growth of Mycobacterium tuberculosis in media containing constant amounts of glucose (10 mg per ml) and varying limiting amounts of ammonium chloride

INITIAL AMMO-NIUM CHLORIDE CONCENTRATION	GENERATION TIME IN HOURS					
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6
mg/ml						
1.0			72	78	34	35
0.1			52–72		30	33
0.025	80	115				
0.02			52–72	76	34	32
0.01	80	110		62	29	37
0.005	80	90				
0.0025	65–70	100				
0.001	50–65					
0	80					

An essential condition for the appearance of autolysis was found to be exhaustion of the nitrogen source in the culture medium. This was proved, not only by the fact that autolysis started in all experiments when the amount of growth had reached a level proportional to the amount of ammonium chloride initially added to the medium, but also by the fact that the addition of a nitrogen source to an autolyzing culture stopped autolysis and initiated new growth (figure 5).

The influence of glucose on autolysis was shown by experiments in which the bacilli were allowed to grow in the presence of a limiting amount of ammonium chloride and of varying initial concentrations of glucose. In order to measure the intensity and the extent of autolysis, the time required for a decrease of 33 per cent in optical density was calculated,[7] and also the loss in percentage of optical density, observed 26 days after the beginning of autolysis. Table 2 gives the values that were obtained in three different experiments. Autolysis was

[7] Turbidimetry does not measure the absolute amount of autolysis since autolyzed bacilli are not optically empty. However, it may be used as an index of the relative amount of autolysis occurring under different conditions.

rapid and showed only small variations for initial glucose concentrations of 2.5 to 10 mg per ml, but was much slower and became insignificant when the initial glucose concentrations were further reduced. The presence of glucose in a relatively high concentration seems to be necessary for the initiation of rapid lysis.

Effect of various concentrations of glucose on tubercle bacilli suspended in a nitrogen-free medium. Cultures of exponentially growing tubercle bacilli were distributed in tubes, centrifuged, and washed twice with the basal medium.[3] The sediments were resuspended, and to some of the suspensions no nutrient was added; to others, glucose or glycerol in various concentrations but no nitrogen
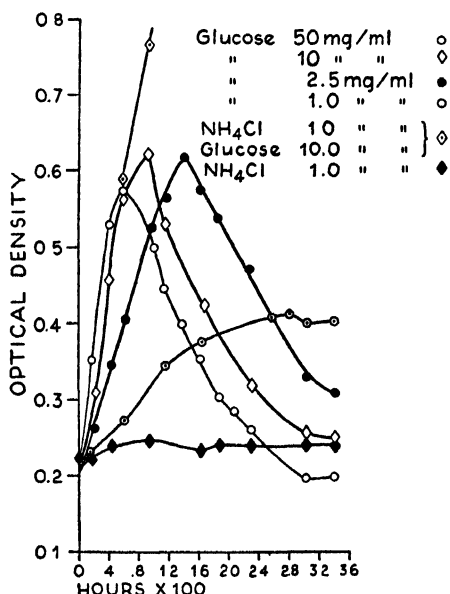
Figure 7. Effect of the addition of glucose, ammonium chloride, or both on washed suspensions of tubercle bacilli taken in exponential phase of growth. The bacteria were washed twice with sorbitan monooleate, albumin, salt mixture prior to the addition of the nutrient.

source; to others, ammonium chloride but no carbon source; to still others, both nutrients. Parallel experiments were made in which albumin was omitted. All suspensions were incubated in the usual way (figure 7).

Neither growth nor lysis was observed when ammonium chloride and glucose were omitted. In the absence of glucose, but with ammonium chloride present, there was likewise no growth and no lysis. When ammonium chloride was absent and a carbon source present, the optical density of the suspension increased by a factor of 3 in the following 2 to 8 days. Then autolysis followed and reduced the optical density nearly to its original level during the succeeding 8 to 10 days. The rate of growth was maximal with glucose concentrations of 10 mg per ml and

[3] The mineral solution of the basal medium contained 0.005 per cent ferric chloride instead of ferric ammonium citrate in order to eliminate this nitrogen source.

above and progressively lower for smaller glucose concentrations. The rate of autolysis was rapid and nearly the same for glucose concentrations of 2.5 mg per ml and above; for smaller glucose concentrations it was minimal. With 5 mg per ml glycerol, the rate of growth was lower than with 2.5 mg per ml glucose, but the rate of lysis was identical. The presence or absence of albumin in the suspension fluid had no influence on the course of events.

From this experiment it follows that for autolysis to occur, the medium must not only be deficient in nitrogen but must also contain a suitable carbon source. It further shows that, when nitrogen was absent from the medium,[9] the optical density nevertheless increased to 3 times the initial amount, provided glucose or glycerine was present in a concentration suitable for growth.

Microscopic examinations were made of the bacteria grown in the presence of an excess of the nitrogen source and in a nitrogen-deficient medium. Under conditions of nitrogen starvation, bacteria showed a trend to increase in length and width. Most striking were the differences in the aspect of the bacteria when stained by the Ziehl-Neelsen procedure. The bacteria grown in the presence of an excess of the nitrogen source were colored deep red, and no details of the inner structure of the bacteria could be recognized. On the contrary, the bacteria grown in the absence of an external nitrogen source were colored pink and showed in their pink cytoplasm the presence of one, two, and sometimes more deeply colored red or blue granules. When the slides were treated by N HCl for 5 minutes at 60 C and then stained by Ziehl's carbol fuchsin, Giemsa, methylene blue, or gram stain, these granules were stained but the cytoplasm had lost its tinctorial affinity.[10] This behavior is considered to be characteristic for nuclear elements (Robinow, 1941). Sometimes these granules formed pairs as if they were split by binary fission. In the final stage of pronounced autolysis, the tinctorial affinity of the cells was almost completely lost.

DISCUSSION

Glucose concentration and the growth rate. It was found that *Mycobacterium tuberculosis* differs from other bacteria by the fact that its growth rate depends on the concentration of glucose in the medium and that the maximal growth rate is reached only when the glucose concentration in the medium is about 10 mg per ml. This behavior suggests that glucose is taken up by *Mycobacterium tuberculosis*, not through an active metabolic process, but by diffusion, the speed of which depends on the gradient of the glucose concentration on the outside and the inside of the cell membrane. The slow diffusion of glucose in the tubercle bacillus would indicate that its cell membrane is relatively impermeable to glucose.

The necessity of a high glucose concentration for maximal growth rate could

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[9] The hypothesis that this increase of optical density is due to an external nitrogen source can be discarded since previously mentioned experiments showed that the basal medium did not permit an amount of growth exceeding 0.1 in optical density. In the experiment under discussion, the increase in optical density was 0.4.

[10] Acid fastness was lost by this treatment.

also be due to the fact that the enzymes of the tubercle bacillus might possesss a very low affinity for glucose and consequently would be saturated only at a relatively high concentration of substrate. This hypothesis, however, seems less probable since glucose was found to be the carbon source that gave the highest growth rate of all nitrogen-free carbon sources tested.

Glucose concentration and yield. The maximum yield in dry weight of bacteria in these experiments was 25 per cent of the glucose added. Monod (1942a) found for the growth of *E. coli, B. subtilis,* and *Salmonella typhimurium* on glucose a constant yield of 25 per cent, Terroine and Wurmser (1922) in the growth of *Aspergillus* a yield of 42 per cent, and Sperber (1946) with yeast grown on an ammonium salt and glucose a yield of 48 per cent. From these data it appears that the yield of *Mycobacterium tuberculosis* resembles that of the other three bacteria mentioned and not that of the fungi.

Nitrogen source and growth. Mycobacterium tuberculosis was able to build up all its nitrogenous cell constituents from an inorganic ammonium salt. The amount of growth was found to be proportional to the initial concentration of this nitrogen source. Cultures that were grown in the presence of optimal amounts of the carbon and the nitrogen source and that were then washed and suspended in a medium containing optimal amounts of the carbon source but no nitrogen source tripled their optical density. This increase of the optical density could be due to swelling, to storage of glycogen, or to cell division without synthesis. It could also be due to real growth, that is, to synthesis of new bacterial substance which could either increase the size of the individual cells without increasing their number or increase the number of the cells without changing their size. This growth in the absence of an external nitrogen source could be imagined only under the assumption that the bacteria grown in the presence of an excess of nitrogen had assimilated not only enough nitrogenous material for their maintenance, but also additional nitrogenous material that could be utilized for the synthesis of new bacterial substance. If this is the case, the bacteria brought into conditions of nitrogen starvation and grown further would contain less nitrogen per dry weight than bacteria grown under conditions of an optimal nitrogen supply. These determinations have not yet been made. However, the occurrence of variations in the nitrogen content of bacteria and yeast cells under the influence of nitrogen starvation is a well-known fact. Terroine, Wurmser, and Montané (1922) observed that the nitrogen content of *Aspergillus niger* decreased from 6 to 2.6 per cent under conditions of nitrogen starvation or nitrogen and carbon starvation. Sperber (1946) found that the nitrogen content of yeast cells changed from 9 to 4.5 per cent when the nitrogen source was omitted from the medium. Virtanen and de Ley (1948) observed that the nitrogen content of *E. coli* decreased from 13 to 6.5 per cent when the nitrogen concentration in the medium was decreased below 40 mg N per liter. These authors also showed that the nitrogenous material that accumulated under conditions of an optimal nitrogen supply was utilized for the formation of adaptive enzymes, but that cells grown with a minimal supply of nitrogen did not build up these enzymes—only those that were absolutely essential for life.

Cytological observations made on cultures kept under conditions of nitrogen

starvation showed an increase in the size of the bacterial cells and pictures of complete or incomplete cell division. This suggests that proliferation and synthesis of cell substance actually occurred under these circumstances. Cytological observations further showed a reduction of the basophilia of the bacterial cytoplasm and the appearance of strongly basophilic "nuclear" bodies. Since basophilia of the cytoplasm is generally attributed to the presence of ribonucleic acid (Dubos, 1945; Tulasne and Vendrely, 1947), its disappearance may indicate a consumption of the cytoplasmic ribonucleic acid under the influence of nitrogen starvation. Similar observations are reported by Knaysi and Baker (1947) with *Bacillus mycoides*.

Autolysis. When the extracellular and the intracellular sources of nitrogen were exhausted by the growth of the bacteria but when the carbon source was still present in excess, cell division and growth ceased and a process of autodigestion and disappearance of the cell substance started. This shift of the metabolic balance from synthesis to degradation was evidently conditioned by the fact that the metabolic reactions were still provided with energy by the carbon source. Since the metabolic reactions of the tubercle bacillus are saturated only at a high glucose concentration, the velocity of the autolytic process also depended on the glucose concentration in the medium. The final disintegration and lysis of the bacterial cells may possibly be attributed to the effect of the surface-active agent sorbitan monooleate on a cell membrane that had lost its normal resistance by the autodigestive process.

It is interesting to compare the autolytic process in *Mycobacterium tuberculosis* with that of other bacteria. Monod (1942*b*) observed a very rapid lysis in cultures of *B. subtilis* when the carbon source was exhausted. Exhaustion of the nitrogen source did not have this effect. Toennies and Gallant (1949) recently described autolysis in cultures of *Streptococcus faecalis* when the lysine content of the medium was exhausted. The presence of a high phosphate concentration (0.3 M) was necessary also. It seems that very different conditions are required to induce lysis depending on the bacterial species.

Mycobacterium tuberculosis has generally been considered to be one of the bacteria most resistant to autolysis. A very slow autolytic process in old cultures of *Mycobacterium tuberculosis* was described by Steenken (1938) and by Laporte (1942*a,b*). Laporte showed that this process required the presence of oxygen and concluded, therefore, that enzymatic processes were involved. The lytic process could be induced artificially by toluol and other fat solvents (Corper and Sweany, 1918; Laporte, 1942*a,b*; Baisden and Yegian, 1943). The lytic filtrate did not induce lysis in other cultures. The partial lysis of *Mycobacterium tuberculosis* in Dubos medium in the presence of high concentrations of penicillin was described by Kirby and Dubos (1947) and attributed to the combined effect of penicillin and the surface-active agent.

SUMMARY

A method is described that permitted the quantitative measurement of the diffuse growth of *Mycobacterium tuberculosis* in sorbitan monooleate medium.

This method was applied to the study of the growth of *Mycobacterium tuber-*

culosis as a function of its carbon and nitrogen sources. In the presence of an ammonium salt and glucose, the growth rate depended on the initial concentration of glucose in the medium and reached its upper limit at a concentration of 10 mg per ml.

When glucose was present in the medium in small amounts, it became the limiting factor for growth. The maximum bacterial yield in dry weight was 25 per cent of the weight of the glucose supplied.

When ammonium nitrogen was present in limiting amounts and glucose was in excess, the total amount of bacterial growth was proportional to the initial concentration of the ammonium salt. When maximal growth was reached under these conditions, autolysis of the bacteria followed at a more or less rapid rate depending on the initial glucose concentration in the medium. Experiments designed to analyze this phenomenon suggested that the bacteria, which were growing in the presence of limiting amounts of the nitrogen source and an excess of the carbon source, continued proliferation not only until the nitrogen source in the medium was depleted but also for at least one generation beyond the point of nitrogen exhaustion.

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THE NATURE OF THE COENZYME OF ASPARTIC ACID, SERINE, AND THREONINE DEAMINASES[1]

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We have reported previously that biotin, as well as muscle adenylic acid, is somehow concerned in the reversible deamination of aspartic acid, and in the deamination of serine and threonine (Lichstein and Umbreit, 1947a; Lichstein and Christman, 1948). This has been demonstrated by employing an aging technique consisting of the exposure of living bacterial cell suspensions to molar phosphate at pH 4 for a short time (Lichstein and Umbreit, 1947a,b; Lichstein and Christman, 1948).

The intimate relationship of biotin and adenylic acid to aspartic acid deaminase has been extended to partially resolved cell-free preparations. It has been shown also that yeast extract is approximately 100-fold more active in the stimulation of partially resolved aspartic acid deaminase than could be accounted for by its assayable biotin content. Studies with such preparations suggested that the preformed coenzyme of aspartic acid deaminase exists in yeast extract and that both biotin and adenylic acid are somehow concerned in its formation (Lichstein, 1949).

METHODS

The organisms employed in these studies were *Bacterium cadaveris* (Gale) and *Proteus vulgaris*, which were grown for approximately 16 hours at 30 C in a medium composed of 1 per cent each of tryptone and yeast extract and 0.5 per cent K_2HPO_4. The cells were harvested by centrifugation, washed once with distilled water, and suspended in M phosphate, pH 4, to give about 1 mg of bacterial nitrogen per ml. Resolution was obtained as previously described by holding the cell suspensions at pH 4 in phosphate buffer for 30 to 60 minutes at 25 to 30 C. The deamination experiments were performed in phosphate buffer at 37 C. After incubation in the presence of an amino acid substrate the reaction was stopped by the addition of 6 molal trichloroacetic acid, and ammonia was determined colorimetrically (Klett-Summerson photoelectric colorimeter) on aliquots of the centrifuged samples. The increase in ammonia over identical samples incubated without added substrate was taken as an index of deamination. The biotin employed was the free acid,[4] the adenylic acid was the adenosine-5-phos-

phoric acid,[5] and the yeast extract was the desiccated product obtained from the Difco Laboratories, Inc.

Microbiological assays for biotin were made with a variety of strains of *Saccharomyces cerevisiae*[6] employing the medium of Snell *et al.* (1940) modified by the addition of 100 μg of nicotinic acid and 20 μg of *para*-aminobenzoic acid per liter. Total growth was measured by nephelometry, and the biotin content was determined by suitable standard curves.

Special techniques employed in the separation of the coenzyme from yeast extract are given in the experimental portion of the paper.

EXPERIMENTAL RESULTS

The data already presented (Lichstein, 1949) indicated the existence of a substance in yeast extract which stimulates aspartic acid deaminase in aged bacterial suspensions and partially resolved cell-free preparations. The data
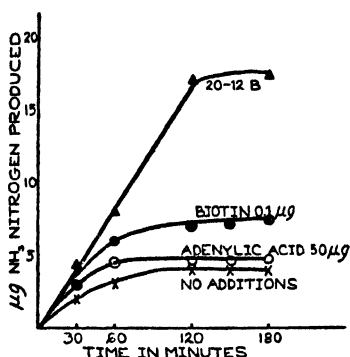
Figure 1. Effect of yeast extract, biotin, and adenylic acid on aspartic acid deaminase in *Bacterium cadaveris.* Cells aged as described in text. Reaction run at pH 4 in 0.5 M phosphate, 37 C. Substrate concentration 0.00125 M (17.5 μg ammonia nitrogen available). Yeast extract (20-12 B) 10 mg per tube.

given in figure 1 demonstrate perhaps more clearly the effect of this material, as well as biotin and adenylic acid, on aged cell suspension of *Bacterium cadaveris.* It may be seen from this figure that the stimulation produced by yeast extract continues linearly to completion. However, that produced by biotin or adenylic acid levels off soon after the initial activation. This phenomenon varies somewhat with the degree and type of resolution obtained by the aging process, and may be due to a deficiency of the enzyme system necessary for the conversion of the coenzyme.

With the demonstration of the presence in yeast extract of a substance that stimulates aspartic acid deaminase, it appeared desirable to attempt concentration, purification, and characterization of this material.

Stability of the stimulating material. Although ignition and oxidation resulted

[5] Kindly supplied to us by the Ernest Bischoff Company, Ivoryton, Connecticut.

[6] These strains were furnished by Hoffmann-LaRoche, Inc., Red Star Yeast and Products Co., Milwaukee, and Joseph E. Seagram and Sons, Inc., Louisville.

in complete inactivation, the material was quite stable to most physical and chemical manipulations. It remained active on heating at 110 C for 3 days, on repeated solution and evaporation to dryness at 100 C, on autoclaving at 121 C for 1 hour, on boiling with 5 per cent HCl or NaOH for 1 hour, and on refluxing with a variety of solvents for long periods of time.

Solubility characteristics. One gram of desiccated Difco yeast extract was suspended in 100 ml of solvent in a 250-ml Erlenmeyer flask, heated to boiling, cooled, and filtered. The filtrate and precipitate were both dried, and a 1 per cent aqueous solution of each was prepared and tested for activity in the stimulation of aspartic acid deaminase in aged cell suspensions of *Bacterium cadaveris*. The active material was insoluble in ether, absolute ether, ethyl acetate, *n*-butyl alcohol, chloroform, and carbon tetrachloride; it was only slightly soluble in *iso*-propyl alcohol; it was relatively soluble in water, methyl alcohol, and 95 per cent and absolute ethyl alcohol. However, no clear-cut separation was achieved with these solvents.

Using absolute ethyl alcohol, we then turned to continuous extraction of yeast extract with the hope of obtaining a more clear-cut separation. Although this procedure resulted in a product that had certain advantageous features, such as lack of hygroscopic nature, removal of a certain amount of ammonium ion, etc., the ratio of the amount of coenzyme to the total amount of material was not appreciably increased.

Adsorption. Adsorption of the active material from dilute solutions at pH 4 by activated carbon, and elution with ammoniacal ethanol, effected considerable purification, but the procedure was unsuitable for use on a large scale in our laboratory.

Paper partition chromatography. The technique employed was essentially that described by Horne and Pollard (1948). In general, 10 to 20 mg of yeast extract were dissolved in a minimum quantity of water and placed 2 inches above the end of the strip in a narrow band. The position was marked, and the strip hung in a chamber with about 5 mm of the end of the strip immersed in the solvent. After a period of time, depending on the solvent, when the solvent front had advanced about 250 mm, the strip was removed from the chamber and dried at 110 C. After drying, the strip was cut into 10 equal pieces and eluted with a constant volume of water, giving approximate concentrations equivalent to an original 1 per cent solution of yeast extract. The samples were then tested for biotin content with *Saccharomyces cerevisiae* (139) and for the ability to activate aspartic acid deaminase in aged cell suspensions of *Bacterium cadaveris*.

The first system studied, that is, water as the mobile phase in a saturated water atmosphere, gave no separation of biotin from the coenzyme and was thus unsatisfactory. This was likewise true of a butanol-water system. To date, the most successful procedure has been the use of a 75 per cent phenol and 25 per cent water mobile phase in a water-saturated atmosphere at 30 C. Because of the involvement of biotin in serine and threonine deaminases, as well as in aspartic acid deaminase (Lichstein and Umbreit, 1947a; Lichstein and Christman, 1948), the eluted samples were tested for the ability to stimulate all three deam-

inases in aged cell suspensions of *Bacterium cadaveris* and for biotin content by stimulation of growth of *Saccharomyces cerevisiae* (139). The results of one such experiment are given in figure 2.

It can be seen from this graph that biotin moves to the top of the chromatogram having an R_f value between 0.8 and 1.0. The stimulatory material for aspartic acid, serine, and threonine deaminases falls between R_f 0.3 and 0.5, with maximum concentration at R_f 0.4. These data show that the activator of
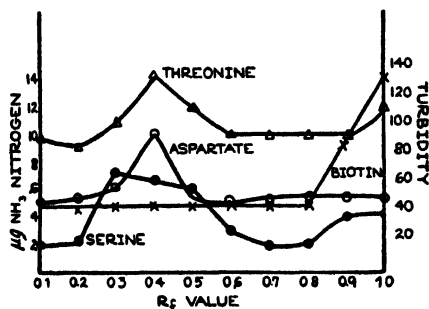
Figure 2. Paper strip chromatogram distribution of biotin and coenzyme from yeast extract. Phenol-water system.
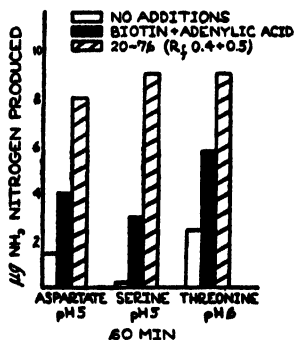
Figure 3. Relative stimulatory effect of biotin plus adenylic acid, and coenzyme from yeast extract on aspartic acid, serine, and threonine deaminases in *Bacterium cadaveris*. Per ml reaction volume, the following concentrations were employed: biotin, 0.1 μg; adenylic acid, 50 μg; coenzyme fraction, 0.1 ml of R_f 0.4 and 0.5 eluted to a total volume of 4 ml.

all three enzymes falls on approximately the same spot and suggest that they are either closely related chemically or identical. It is probably that the material is relatively pure with respect to free biotin as assayed by *Saccharomyces cerevisiae* (139), and further that this organism does not respond to the coenzyme fraction.

The relative stimulatory effect of a pooled sample composed of R_f 0.4 and 0.5 on aspartic acid, serine, and threonine deaminases in *Bacterium cadaveris* is shown in figure 3. Although the involvement of biotin in these deaminases does not necessarily mean that it is the coenzyme or even part of the coenzyme, we proceeded to determine whether or not biotin could be detected in the coenzyme

fraction separated from whole yeast extract by chromatography employing the phenol-water system.

As already stated, *Saccharomyces cerevisiae* (139) does not respond to the coenzyme fraction. This presents two possibilities, either (1) there is no biotin in this fraction, or (2) the biotin is not available to this strain. The existence of bound forms of biotin, that is, forms that may be microbiologically unavailable, has been demonstrated by many investigators (Bowden and Peterson, 1949).

Experimentally we attacked the problem from two directions: (1) a search for organisms that might respond to the coenzyme fraction in a biotin-free medium, and (2) hydrolysis of the coenzyme fraction to determine whether

TABLE 1

Relative effect on growth of two strains of Saccharomyces cerevisiae by yeast extract after one-step purification on phenol-water chromatogram

SAMPLE	TURBIDITY	
	S. cerevisiae 139	*S. cerevisiae* Java
No additions...	30	30
Biotin 10^{-5} µg..	32	33
Biotin 10^{-3} µg.	90	90
Biotin 10^{-1} µg	239	154
Biotin 10^{1} µg .	242	160
Pooled R_f 0.1, 0.2, 0.6, 0.7, 0.8	35	40
Same hydrolyzed* .	28	32
Pooled R_f 0.3, 0.4, 0.5	35	65
Same hydrolyzed* .	50	48
Pooled R_f 0.9, 1.0	90	85
Same hydrolyzed*	26	25
Biotin 1 µg	260	175
Same hydrolyzed*	145	140

* 6 N H_2SO_4, 121 C, 2 hours.

biotin may then be made available to *Saccharomyces cerevisiae* (139). The results of such an investigation are given in table 1. It is noted here that *S. cerevisiae* (Java) responds to the coenzyme fraction, but *S. cerevisiae* (139) does not. Further, hydrolysis of the coenzyme fraction liberates biotin for *S. cerevisiae* (139), which does not respond to the unhydrolyzed material. The fact that bound biotin occurs in large amounts only in the fraction containing the coenzyme may be interpreted in two ways, either (1) the coenzyme is a bound form of biotin, or (2) the relationship is due to contamination of the coenzyme with bound biotin of yeast extract and upon further purification the bound biotin may be separated from the coenzyme. It appeared that this problem could be clarified by further purification of the coenzyme fraction. This was achieved by rerunning only the coenzyme fraction of 38 strips through two further chromatograms as

follows: The R_f 0.4 to 0.6 section of each strip was eluted with hot water; the eluants were combined and evaporated to dryness under infrared light. The material was redissolved in a minimum quantity of water and divided among seven strips, which were then run in the phenol-water system. The coenzyme fractions (R_f 0.4 to 0.6) of these strips were eluted similarly, and after evaporation rerun on one strip.
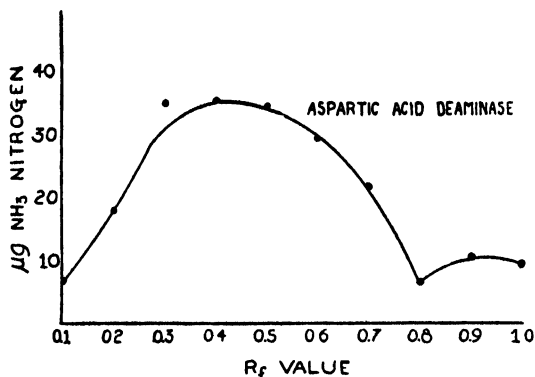
Figure 4. Paper strip chromatogram distribution of coenzyme assayed against aspartic acid deaminase in *Bacterium cadaveris*. Three-step purification. Phenol-water system.
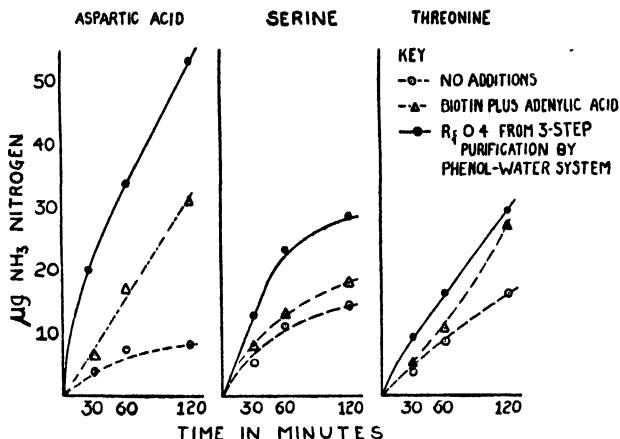
Figure 5. Relative stimulatory effect of biotin plus adenylic acid, and coenzyme from yeast extract (three-step purification on phenol-water system) on aspartic acid, serine, and threonine deaminases in *Bacterium cadaveris*. Concentration of stimulatory materials as in figure 3.

The distribution of the stimulatory material for aspartic acid deaminase in aged cell suspensions of *Bacterium cadaveris* shows a wider spread than previously and is probably due to greater concentration (figure 4). However, the greatest concentration of material is at R_f 0.3 to 0.5. The relative stimulatory effect of the R_f 0.4 fraction on partially resolved aspartic acid, serine, and threonine deaminases in *Bacterium cadaveris* as compared with the effect of biotin and adenylic acid may be seen in figure 5.

A pooled sample containing R_f 0.3 to 0.6 was assayed against the 139 Java strains of *S. cerevisiae* in order to determine the bound biotin content of the coenzyme fraction. In addition, a duplicate set of tubes were held in boiling water for variable periods of time to ascertain whether or not free biotin could be liberated. These results are given in table 2.

TABLE 2

Relative effect on growth of two strains of Saccharomyces cerevisiae by yeast extract after three-step purification on phenol-water chromatogram

SAMPLE	TURBIDITY			
	S. cerevisiae 139		*S. cerevisiae* Java	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
No additions	22	22	26	54
Biotin 10^{-5} μg . . .	44	55	50	74
Biotin 10^{-3} μg	103	135	102	115
Biotin 10^{-1} μg .	210	278	155	216
Biotin 10^1 μg . . .	220	300	146	212
R_f 0.3, 0.4, 0.5	30	21	50	75
Same, boiled 1 hour	28	38	52	125
Same, boiled 2 hours	26	80	65	105
Same, boiled 3 hours .	42	29	72	90

TABLE 3

Titration of coenzyme fraction from three-step purification of yeast extract on growth of two strains of Saccharomyces cerevisiae

SAMPLE	TURBIDITY	
	S. cerevisiae 139	*S. cerevisiae* Java
No additions . .	20	37
Biotin 10^{-5} μg . . .	63	75
Biotin 10^{-3} μg . .	105	105
Biotin 10^{-1} μg	209	160
Biotin 10^1 μg . . .	219	155
R_f 0.3, 0.4, 0.5—0.10 ml	22	105
0.08 ml	—	96
0.04 ml . .	—	76
0.02 ml..	—	85
0.01 ml	—	65

It may be noted that once again the 139 strain does not respond to the coenzyme fraction, whereas growth of the Java strain is supported by this fraction in a biotin-deficient medium. It may be seen also that gentle hydrolysis of the coenzyme fraction, boiling in water rather than with 6 N sulfuric acid, liberates free biotin, which is assayed by both organisms.

The absence of free biotin in the coenzyme fraction is more clearly demon-

strated by the data given in table 3. It may be noted here that *S. cerevisiae* 139 fails to show any response to the fraction, whereas the Java strain exhibits excellent growth in several dilutions.

These results lead one to the conclusion that bound biotin is firmly associated with the coenzyme of these deaminases. Unfortunately, the amount of coenzyme separated from yeast extract by the methods employed has prevented more quantitative data, so that we are not at present in a position to determine the absolute ratio of bound biotin to coenzyme. Further, we recognize that we do not have a pure material, but rather have effected considerable purification and more particularly a separation of the coenzyme from free biotin. More recent results with a four-step purification, in which 2 grams of yeast extract were concentrated to 27 milligrams, by titration studies revealed a parallelism between coenzyme activity and the bound biotin content of the concentrate, since the highest dilution exhibiting coenzyme activity was identical with the highest dilution sufficient to support growth of *S. cerevisiae* (Java).

ACKNOWLEDGMENT

SUMMARY

By means of paper strip chromatography we have been able to separate from yeast extract a substance or substances that activate the deaminases of aspartic acid, serine, and threonine. The material is not adenylic acid or biotin as such, and from the nature of the stimulations obtained it is concluded that this fraction contains the coenzymes of these reactions. From the position on the chromatogram it is probable that they are chemically similar or identical.

Although the coenzyme fraction is not pure, it has been purified with respect to free biotin. This fraction supports the growth of *Saccharomyces cerevisiae* (Java) in a biotin-deficient medium, and upon hydrolysis biotin is made available to *Saccharomyces cerevisiae* (139), which does not respond to the unhydrolyzed material.

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THE PASSAGE OF MICROORGANISMS THROUGH THE DIGESTIVE TRACT OF BLABERUS CRANIFER MOUNTED UNDER CONTROLLED CONDITIONS

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The technique of mounting insects and controlling their food intake was discussed by Wedberg and Clarke (1947) in an earlier report on this subject Subsequent investigation has resulted in certain improvements in the apparatus, and the more recent developments in this technique are shown in figure 1.

Mounting the roaches on wax blocks resting directly on the lids of Coplin jars rather than on a glass swivel rod eliminates some of the difficulties encountered when the room temperature rises sufficiently to soften the wax blocks. The multiple layers of cheesecloth employed in our earlier work to eliminate contamination in our battery jars have been replaced with glass lids, and a sheet of window glass separates the two halves of the jar when two insects are to be studied simultaneously. The lids on these jars are loose enough to allow ready access of air. Since some of the work was conducted with pathogenic organisms, a 1:1,000 solution of mercuric chloride was placed in the bottom of the battery jars to serve as a disinfectant for any of the pathogens finding their way there.

The need for an inventory and systematic cataloguing of the bacteria associated extracellularly with insects and ticks was pointed out by Steinhaus (1946). Since the information relative to *Blaberus cranifer* has received little attention in this respect, experiments have been conducted to determine the normal flora of these roaches, and subsequent investigations have been directed toward attempting to implant organisms in the digestive tract of these insects If certain insects are capable of serving as passive carriers of pathogenic bacteria, or if they are capable of actually permitting the multiplication of disease-producing organisms within their digestive tract, it is conceivable that, under a proper set of conditions, certain insects might be in a position to initiate epidemics, particularly of the food-borne type.

Much of our present literature is based upon uncontrolled experiments, and the significance of some of the findings is questionable. By mounting the insects, feeding them a controlled diet, and determining the flora of the passed excreta, investigators should obtain more valid qualitative and quantitative results. Roaches caught in hospital wards in Brisbane, Australia, during an epidemic of gastroenteritis caused by salmonellae were found by Mackerras and Mackerras (1948) to harbor the specific organisms causing the epidemic. Abundant infective material was available in the sinks or tubs in which diapers were rinsed before being sent to the laundry. This suggests a potential means of infecting the food of adults or older children. Bitter (1949) isolated *Salmonella schottmuelleri*, *Salmonella oranienburg*, and *Salmonella bredeney* during the course of her studies

of 94 specimens of the American roach, *Periplaneta americana*, collected primarily from sewer manholes in Texas.

LONGEVITY STUDIES

Much of our own research has been conducted during the colder months of the academic year, and, since the source of insect supply was located in Florida, it was found that allowing the colony of roaches an adjustment period of about a week to feed on "pablum" and water appeared to minimize the death rate of the mounted insects during the first week of actual study. The position of the roaches,
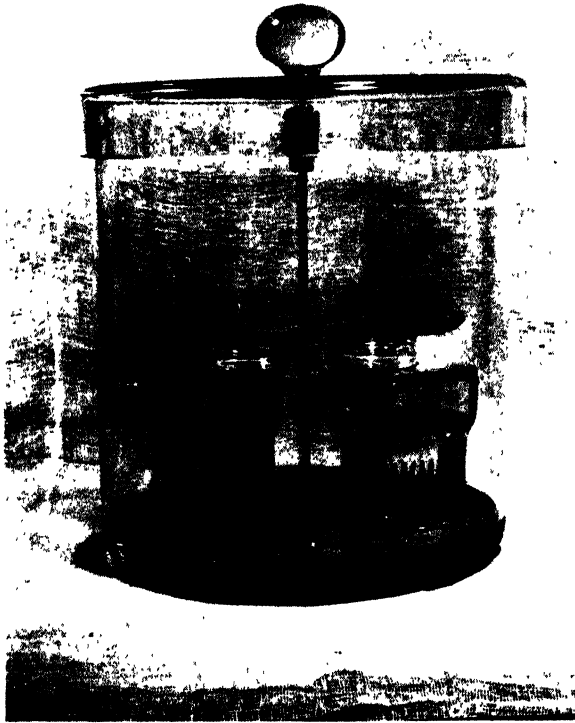
Figure 1. The method of storing the insects under controlled conditions.

light, temperature, and humidity did not appear to have a marked influence on the survival time. But for practical reasons the insects have been mounted in a horizontal position, which reduces the chances of body contaminants gaining access to the material being cultured.

A study was undertaken to determine which basic diet would sustain the mounted insects for a long enough time to allow extended observations. As in the previous work, each roach was fed individually with the aid of sterilized needles and syringes. Since Penner (1947) had reported the survival of mounted flies for several months on nothing more than molar sucrose solution, one group of 10 roaches was fed on this same sterile diet. Five of these insects survived for from

39 to at least 75 days. The 75-day roach was still alive at the conclusion of that experiment. The remaining roaches lived for 4, 12, 14, 15, and 19 days. One of the major difficulties encountered with this diet, however, was the irregularity of excretion coupled with the minuteness of the excreta when it was passed. Mackerras and Pope (1948) reported that roaches fed on sucrose tended to become bloated, probably from gas, and some of our results confirmed this finding.

Skim milk when combined with Difco yeast extract (1 per cent) helped somewhat to overcome the difficulties encountered with sucrose alone, and the survival time of the 17 roaches tested was as high as 86 days. However, the combination of skim milk, 0.5 per cent sucrose, and 1 per cent Difco yeast extract yielded survival times as long as 145 days in one instance and 92 days in another. Since the average survival time of the 10 roaches tested was 51 days, we chose this combination of food as the basic diet in subsequent studies. Droppings were passed more regularly and the volume of excreta seemed to increase over the amounts obtained when the other diets were fed. A period of sluggishness and loss of appetite usually preceded the death of the mounted insects.

METHODS

As soon as a roach had been placed on a paraffin block, small glass dishes containing saline were placed in position to catch the excreta. After emulsifying the droppings in saline, streak plates were made with standard nutrient agar, tomato agar, and either Endo agar or Difco SS agar in the preliminary determination of the normal excreta flora of the freshly mounted roaches. No anaerobic studies were made.

The standard nutrient agar plates and the tomato agar plates were incubated at room temperature for periods of at least 5 days and in many instances for 3 weeks, with observations being made daily. The Endo or SS plates were kept at 37 C for 24 to 48 hours before colonies were fished to Kligler's triple-sugar iron medium.

The fecal flora tended to change as the length of the mounting period increased. This is probably to be expected since the diet is more limited and the chance ingestion of many organisms is minimized. The gram-negative rods usually predominated throughout the entire period of controlled study. Strong lactose fermenters tended to be replaced by weak and slow lactose fermenters, and the number of species isolated from a given roach decreased as the mounting period increased. The following organisms were isolated from the droppings of *Blaberus cranifer: Aerobacter aerogenes; Alcaligenes faecalis; Bacillus cereus; Bacillus subtilis; Escherichia coli* var. *communior; Escherichia freundii;* intermediate coliforms; *Micrococcus pyogenes* var. *albus; Micrococcus pyogenes* var. *aureus; Proteus vulgaris; Pseudomonas aeruginosa; Rhizopus nigricans; Penicillium* species; *Saccharomyces cerevisiae;* unidentified cocci; gram-negative rods; molds; yeasts; and actinomyces.

CONTROLLED FEEDING OF ORGANISMS

Serratia marcescens. Hawley (1948) reported that when he fed small numbers of *Escherichia coli, Shigella dysenteriae,* or *Salmonella schottmuelleri* to the house-

fly, *Musca domestica*, he was unable to recover the organisms from the feces of the flies. However, when he fed the flies a suspension of between 12,000 and 48,000 organisms, actual multiplication occurred within the houseflies and the test organisms could be recovered from their droppings. Up to a certain point, the more organisms fed, the more rapid was the multiplication within the flies. Bearing these observations in mind, we undertook controlled feeding of the red-pigmented *Serratia marcescens* since it is relatively easy to detect on streak plates. Even very small numbers of these bacteria when fed to the roaches seemed to establish themselves in the digestive tract, and in one instance we were able to recover them in the stools 143 days after they were fed. Tremendous multiplication of *Serratia marcescens* occurred in some roaches, and at the time of their death the upper half of their bodies and limbs were a deep-red color, visible through the body wall. Streak plates made from these tissues and from the digestive tract revealed practically pure cultures of the pigmented organism. Whether they actually caused the death of the insects is difficult to say, but they might have been a contributing factor in some instances, particularly when the pigment was visible through the body wall. It has been reported by DeBach and McOmie (1939) that their laboratory stock of the termite, *Zootermopsis angusticollis*, was suffering from a disease caused by *Serratia marcescens* in which the head and appendages turned red. In our studies, before any roaches were dissected, the insects were first soaked in 1:1,000 bichloride of mercury for at least a period of 30 minutes, and an aseptic technique was followed throughout.

When a loop of culture was scraped from a plate, emulsified in liquid, and fed to the roaches, there were no instances in which *Serratia* failed to be excreted, usually within 48 hours, and it continued to be excreted periodically throughout the life of the mounted roach. This would indicate that implantation had taken place. The two roaches which lived the longest in the entire series (145 and 92 days,) were both found to be harboring *Serratia marcescens* at the end of the experiment, and the 92-day roach showed an intense reddening of its upper body and its extremities just at the time of death.

Torula rosea. There was no evidence in these studies that the yeast *Torula rosea* was pathogenic for *Blaberus cranifer*, nor were any isolations of *Torula* species made at any time in the studies of normal flora. Even massive doses of this yeast fed as loops of colonies scraped from tomato agar plates failed to shorten the life of the mounted insects. The results appear to indicate that implantation of this yeast is rather difficult, if possible at all. Upon repeated feeding of massive doses, it was possible to isolate the yeast from droppings up to 6 days, but in no instance were we able to isolate the organisms from the roaches after death. *Saccharomyces cerevisiae*, on the other hand, was found to be a natural inhabitant of some roaches and could be isolated from their droppings and also from their digestive tract after death.

Salmonella. Laboratory tests conducted by Olsen (1949) showed that some cockroaches will harbor *Salmonella* species for several days. If the roaches deposit their excreta on food or dishes in the pantry, salmonellae will remain alive a month or so. When small numbers of *Salmonella typhimurium* (less than 5,000

organisms) were fed to *Blaberus cranifer*, they failed to become established in the digestive tract or to be excreted by the mounted roaches. Massive amounts of this organism, however, when fed to the insects, did pass through the digestive tract and were recovered from the excreta in some cases up to 12 days. A decided change in the consistency of the droppings of these roaches was noted. Very moist, liquid excreta were the rule, and the test organisms were readily isolated from this excreta. The only other instances in which we observed such a change in consistency of excreta involved roaches excreting *Pseudomonas aeruginosa* and occasional roaches being fed a diet of sterile sucrose.

Our attempts to pass *Salmonella typhosa* through *Blaberus cranifer* met with failure. Perhaps the enrichment culture technique was not pursued far enough. No significant change in the longevity of the mounted roaches fed massive doses of the typhoid organism was noted, and in no instance were we able to isolate the test organism either from the excreta or from the digestive tract of the insects at autopsy.

STUDIES IN PROGRESS

A limited number of studies have been made with the meal beetle, *Tenebrio molitor*, which might get into food to be used for human consumption. Small doses of *Salmonella typhimurium* fed to these insects failed to establish themselves in the tract, but massive doses when fed were recoverable in the feces after 48 hours. *Aerobacter aerogenes* was a common organism in the feces of these insects during the entire period of their restraint and appears to be a part of their normal flora. *Serratia marcescens* became established when heavy doses were fed. The longevity of the three beetles studied was 13, 15, and 15 days, when they were fed the basic diet of skim milk, 0.5 per cent sucrose, and 1 per cent Difco yeast extract. A few streptococci were isolated from the excreta, but in general the *Aerobacter aerogenes* predominated. No molds or yeasts were isolated.

It is our hope to continue these studies on *Tenebrio molitor* and to begin investigations on the American cockroach, *Periplaneta americana*, and possibly other insects.

SUMMARY

An improvement in the technique for controlled feeding of insects is described. Mounted *Blaberus cranifer* roaches were kept alive for periods up to 145 days when they were fed a basic diet of skim milk, 0.5 per cent sucrose, and 1 per cent Difco yeast extract. Sterile molar sucrose alone maintained the insects for periods up to 75 days.

The normal flora of droppings was determined and followed by controlled feeding of specific organisms in an attempt to implant the organisms in the digestive tract of the roaches. *Salmonella typhimurium* when fed in massive doses appeared to establish itself and to multiply in the roaches. Relatively small numbers of *Serratia marcescens* when fed to the insects resulted in positive excreta cultures within 48 hours, and these organisms were excreted intermit-

tently throughout the life of the mounted insects. In some instances *Serratia marcescens* became so abundant in the body of the roaches that the insects actually turned a deep-red color in the upper half of their bodies and in their extremities. Death always followed shortly after this reddening appeared, and tests made at autopsy always revealed a practically pure culture of the test organism in the colored area.

Attempts to implant *Torula rosea* met with failure, even when massive feedings of the yeast were made. *Saccharomyces cerevisiae*, however, seemed to be a common inhabitant of many of the roaches, and these organisms were excreted intermittently throughout the life of the insects. *Salmonella typhosa* could not be recovered from the droppings of the mounted roaches even though billions of cells were fed repeatedly.

A few preliminary observations on controlled feeding of the meal beetle, *Tenebrio molitor*, are reported.

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GROWTH PROMOTION AND ANTIBIOTIN EFFECT OF HOMOBIOTIN AND NORBIOTIN

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Goldberg *et al.* (1947) and Scheiner and Rubin (1948) have recently reported the antibiotin activity of some homologs of biotin (I, n = 4), namely, homobiotin (I, n = 5) and norbiotin (I, n = 3).

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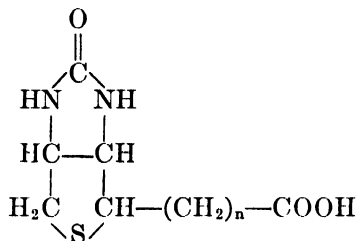
(I)

In the course of studies in this laboratory on the enzymatic functions of biotin, it appeared desirable to make use of the inhibitory effect of these biotin homologs. Repeated attempts to confirm the results of these investigators with a strain of yeast isolated in this laboratory and provisionally called *Saccharomyces cerevisiae* (T) were negative. Through the courtesy of Dr. S. H. Rubin of Hoffmann-LaRoche, Inc., we obtained a transplant of their strain of *Saccharomyces cerevisiae* (139), and the present report concerns our studies on the effect of homobiotin and norbiotin on these and other strains of yeast.

MATERIALS AND METHODS

The organisms employed were *Saccharomyces cerevisiae* (T), *Saccharomyces cerevisiae* (139), *Saccharomyces globosus*, *Saccharomyces fragilis*, and *Zygosaccharomyces barkeri*, which were maintained on a medium composed of 1 per cent each of yeast extract, tryptone, and glucose and 0.5 per cent K_2HPO_4 at 30 C.

The synthetic biotin-free medium was that of Snell *et al.* (1940) with the following additions per liter: 100 μg nicotinic acid and 20 μg *para*-aminobenzoic acid. Assays were set up and run as described by Snell *et al.* (1940), using turbidity measurements as an index of growth.

The biotin, norbiotin, and homobiotin were furnished by Hoffmann-LaRoche, Inc., Nutley, New Jersey.

EXPERIMENTAL RESULTS

The effect of biotin, homobiotin, and norbiotin on the 139 and T strains of *S. cerevisiae* is shown in table 1. The results with the 139 strain are similar to those obtained by Goldberg *et al.* (1947) and Scheiner and Rubin (1948), that

is, failure of growth and inhibition of biotin utilization in the presence of these biotin homologs. In contrast, both homobiotin and norbiotin support growth of *S. cerevisiae* (T) in a synthetic medium free of biotin, and an additive effect on

TABLE I

The effect of biotin, homobiotin, and norbiotin on the growth of S. cerevisiae (139) and S. cerevisiae (T)

ADDITIONS	TURBIDITY	
	S. cerevisiae (T)	*S. cerevisiae* (139)
	21 hr	*21 hr*
None......	20	17
Biotin 10^{-6} μg........	21	17
Biotin 10^{-5} μg..	33	36
Biotin 10^{-4} μg......	70	93
Biotin 10^{-3} μg.......	96	144
Biotin 10^{-2} μg.......	124	167
Biotin 10^{-1} μg.......	122	165
Homobiotin　　2 μg........	71	15
Homobiotin　　20 μg.......	93	8
Homobiotin ...200 μg.......	110	7
Homobiotin　2,000 μg.......	155	9
Homobiotin 20,000 μg........	170	—
Homobiotin　　2 μg + biotin 10^{-3} μg......	127	146
Homobiotin　　20 μg + biotin 10^{-3} μg.......	127	150
Homobiotin　　200 μg + biotin 10^{-3} μg.......	138	127
Homobiotin　2,000 μg + biotin 10^{-3} μg.......	137	13
Homobiotin 20,000 μg + biotin 10^{-3} μg.......	157	—
Norbiotin　　2 μg.......	34	8
Norbiotin　　20 μg.......	75	7
Norbiotin　　200 μg.......	97	9
Norbiotin　2,000 μg.......	140	7
Norbiotin 20,000 μg.......	180	—
Norbiotin　　2 μg + biotin 10^{-3} μg.......	122	133
Norbiotin　　20 μg + biotin 10^{-3} μg.......	135	118
Norbiotin　　200 μg + biotin 10^{-3} μg.......	125	85
Norbiotin　2,000 μg + biotin 10^{-3} μg.......	141	15
Norbiotin 20,000 μg + biotin 10^{-3} μg.......	150	—

growth is suggested when either of these biotin homologs is combined with biotin. There is no evidence of any antibiotin effect with this strain.

Inasmuch as the response of these two strains of *S. cerevisiae* to homobiotin and norbiotin was so different, it seemed advisable to determine the effect of these compounds on the growth of other strains. It may be seen (table 2) that *S. globosus* responds to homobiotin and norbiotin in a manner similar to *S. cere-*

visiae (T), whereas *S. fragilis* and *Z. barkeri* are similar to the 139 strain. Thus, of the five strains studied, three do not utilize these homologs of biotin and biotin utilization is inhibited by these compounds, but two are able to grow in the presence of either homobiotin or norbiotin in a chemically defined medium devoid of biotin.

Concerning the ability of *S. cerevisiae* (T) and *S. globosus* to grow in the presence of these biotin homologs, two possibilities exist: (1) that samples of homo-

TABLE 2

The effect of biotin, homobiotin, and norbiotin on the growth of several strains of yeast

ADDITIONS	TURBIDITY			
	S. fragilis	*S. globosus*	*S. globosus*	*Z. barkeri*
	44 hr	*76 hr*	*44 hr*	*48 hr*
None	24	72	45	8
Biotin 10^{-6} μg	—	—	—	15
Biotin 10^{-5} μg	32	97	62	—
Biotin 10^{-4} μg	79	134	—	—
Biotin 10^{-3} μg	228	161	93	87
Biotin 10^{-2} μg	280	204	—	—
Biotin 10^{-1} μg	280	200	108	83
Homobiotin 2 μg	—	121	—	—
Homobiotin 20 μg	14	170	103	4
Homobiotin 200 μg	18	232	115	5
Homobiotin 2,000 μg	26	232	—	—
Homobiotin 2 μg + biotin 10^{-3} μg	—	165	—	—
Homobiotin 20 μg + biotin 10^{-3} μg	165	197	120	63
Homobiotin 200 μg + biotin 10^{-3} μg	51	234	126	13
Homobiotin 2,000 μg + biotin 10^{-3} μg	53	224	—	—
Norbiotin 20 μg	14	—	60	—
Norbiotin 200 μg	16	—	91	—
Norbiotin 2,000 μg	23	—	—	—
Norbiotin 20 μg + biotin 10^{-3} μg	204	—	101	69
Norbiotin 200 μg + biotin 10^{-3} μg	38	—	111	43
Norbiotin 2,000 μg + biotin 10^{-3} μg	23	—	—	12

biotin and norbiotin are contaminated with sufficient biotin to allow growth, or (2) that homobiotin and norbiotin are utilized as such.

The following data suggest that the ability of certain organisms to grow in the presence of homobiotin and norbiotin is not due to biotin contamination of these compounds. If the amount of growth in the presence of either homobiotin or norbiotin is due to biotin present in the sample, then a given amount of either compound should be equivalent to a constant amount of biotin. It may be seen from the data cited (tables 1 and 2) that 20 μg of homobiotin is equivalent to

TABLE 3

The response of "mutated" S. cerevisiae (T) to biotin, homobiotin, and norbiotin

ADDITIONS	TURBIDITY
	25 hr
None.	22
Biotin 10^{-6} μg	26
Biotin 10^{-5} μg	55
Biotin 10^{-4} μg	90
Biotin 10^{-3} μg	148
Biotin 10^{-2} μg	216
Biotin 10^{-1} μg	254
Biotin 10^{0} μg	300
Biotin 10^{1} μg	290
Homobiotin 2 μg	63
Homobiotin 20 μg	81
Homobiotin 200 μg	147
Homobiotin 2,000 μg	147
Norbiotin 2 μg	35
Norbiotin 20 μg	67
Norbiotin 200 μg.	110

Figure 1. Additive effect of homobiotin and biotin on the growth of *Saccharomyces cerevisiae* (T), 24 hours, 30 C.

10^{-3} μg of biotin for both *S. cerevisiae* (T) and *S. globosus*, whereas 20 μg of norbiotin is equivalent to 10^{-4} μg of biotin. These values have been rather constant and are, of course, suggestive of biotin present in the samples of homobiotin and norbiotin. However, the data in table 3 show that *S. cerevisiae* (T) has under-

gone a change so that the biotin equivalents with the same samples are now: 20 μg homobiotin equals 10^{-4} μg biotin and 20 μg norbiotin equals 10^{-5} μg biotin. Also, whereas in earlier studies (table 1) the homobiotin stimulation of growth continued linearly at least to 20 mg per tube, the data of table 3 show a leveling off of growth at 200 μg of homobiotin. Although the reason for this change in response is not known, the data show that the ability of these compounds to support the growth of certain organisms in a biotin-deficient medium is not because of biotin contamination, but rather because of a direct utilization of these compounds. Further, the additive effect of these compounds plus biotin on the growth of *S. cerevisiae* (T) as seen in tables 1 and 3, and perhaps more clearly in figure 1, is additional evidence against biotin contamination of homobiotin and norbiotin. If the additive effect were due to biotin present in samples of these compounds, one should expect the effect to decrease as the biotin concentration is increased. However, one finds a constant additive effect of homobiotin plus biotin (figure 1).

It would therefore appear that, whereas both homobiotin and norbiotin exhibit antibiotin activity against certain organisms, for others these compounds may replace the need for biotin in a chemically defined medium. Attempts to determine whether homobiotin and norbiotin are utilized as such or converted to biotin have not been successful because of the liberation of other growth-promoting substances upon the hydrolysis of cells grown in the presence of these compounds. Two types of data suggest that the replacement of biotin by these homologs is not because of their conversion to biotin. First, as seen in tables 1 and 2, for *S. cerevisiae* (T) and *S. globosus* the growth response in the presence of either homobiotin or norbiotin may exceed that produced by biotin in the same synthetic medium. Secondly, after 48 hours' incubation at 30 C the growth of *S. cerevisiae* (T) in the deficient medium and in the deficient medium plus homobiotin or norbiotin is characteristic of that produced by organisms unable to utilize atmospheric oxygen, that is, the growth is entirely limited to the bottom, with a clear medium on top. In sharp contrast, the growth in the basal medium plus biotin exhibits a heavy sediment and a heavy surface film. It would appear from this observation that, although homobiotin and norbiotin may replace the need of biotin for growth, they do not replace all of the functions of biotin.

SUMMARY

Data are presented to show that, although homobiotin and norbiotin exhibit antibiotin activity against certain strains of yeast, for others these compounds may replace biotin for growth.

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ANTAGONISM TO SULFATHIAZOLE BY METHYLENE BLUE AND RIBOFLAVIN IN PNEUMOCOCCAL RESPIRATION[1]

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In a previous study (Sevag and Gots, 1948) on the effects of various drugs on the pneumococcal dehydrogenase systems it was found that, whereas acriflavine, atabrine, propamidine, and optochin could inhibit these systems, high concentrations of sulfonamides had no effect. Other investigators (Mellon and Bambas, 1937; Brazda and Rice, 1942; Fox, 1942; Clifton and Loewinger, 1943) have reported the failure of sulfonamides to inhibit dehydrogenase systems. However, sulfonamides have been shown (Barron and Jacobs, 1937; Chu and Hastings, 1938; Dorfman *et al.*, 1940, 1942; Sevag *et al.*, 1942, 1945) to have a marked effect in inhibiting the aerobic respiration of various organisms. This apparent inconsistency between the effect of sulfonamide on aerobic respiration as measured manometrically and dehydrogenase activity as measured, usually, by methylene blue reduction required experimental elucidation.

Some of these inconsistencies were interpreted (Sevag, 1946) to indicate that methylene blue competed effectively with sulfonamide for flavoprotein and therefore no inhibition of methylene blue reduction could be observed. The data presented here and elsewhere (Sevag and Gots, 1948; Sevag, Gots, and Steers, 1950; Gots and Sevag, 1949) show that methylene blue does antagonize the inhibition of aerobic respiration of pneumococcus, type I, by sulfathiazole, and that, under certain conditions, riboflavin has similar effects. It was also found that inhibition by sulfathiazole of methylene blue reduction can be achieved in systems where the sulfonamide is allowed to react with the cells in the absence of the protecting methylene blue.

EXPERIMENTAL METHODS AND RESULTS

Inhibition of methylene blue reduction. The organism used was pneumococcus, type I, in the mucoid phase. Its maintenance and the culturing and preparation of resting cell suspensions for use in these experiments have been described previously (Sevag and Gots, 1948; Gots and Sevag, 1948).

Dehydrogenase activity was measured by means of the conventional Thunberg technique. The test systems, after mixing, consisted of 1 ml of bacterial suspension (1 mg of cells per ml), 0.5 ml of 0.002 M methylene blue solution (in M/20 phosphate buffer at pH 7.6), giving a final concentration of 0.00025 M in the system, 1 ml of substrate (glucose—final concentration, 0.001 M), and either

1.5 ml of phosphate buffer (M/20 at pH 7.6) or 1.5 ml solution (150 mg per cent) of sulfathiazole (final concentration, 0.0022 M) to give a final volume of 4 ml. The reduction of methylene blue in the presence and absence of sulfathiazole was studied by varying the order of the addition of various components. The components in the tubes were in contact with one another for a period of 30 minutes before the contents of the side arm were introduced. This represented the period required for the filling and closing of the tubes, evacuation by a high vacuum pump through a manifold to ensure uniform evacuation (5 minutes), and equilibration (10 minutes) to the temperature of the water bath (37 C). The reactive system consisting of organisms, substrate, and indicator were separated in all systems so that reduction would not begin until mixing. Zero time was taken at the time of mixing, and the time required for 100 per cent reduction of the methylene blue was recorded.

TABLE 1

Effect of sulfathiazole on the reduction of methylene blue by pneumococcus, type I, in the presence of glucose

COMBINATION	CONTENTS OF			REDUCTION TIME (MINUTES)	
	Side arm	Tube		Exp. 1	Expt. 2
A	Glucose	Pneumococci + methylene blue } +	Buffer Sulfathiazole	19 21	22 23
B	Pneumococci	Methylene blue + glucose } +	Buffer Sulfathiazole	18 20	20 21
C	Methylene blue	Pneumococci + glucose } +	Buffer Sulfathiazole	22 42	23 40

For the details of the systems see the text.

From table 1 it can be seen that sulfathiazole inhibited (45 per cent) the reduction time only when it was allowed to react for 30 minutes with the organisms in the absence of methylene blue (combination C). When sulfathiazole was in contact with the organisms in the presence of methylene blue (combination A), no inhibition was observed. Likewise, when the organisms were in contact with neither of the reagents but were introduced so that they reacted simultaneously (combination B), no inhibition occurred. Methylene blue alone exercised no demonstrable inhibition during the exposure of the organisms to it, as can be seen by comparing the control activities of systems A and B. Thus, sulfathiazole can inhibit methylene blue reduction only when it is allowed to react with the organisms in the absence of the protecting methylene blue.

Similar results were obtained with glycerol as substrate; but, since six times as many organisms were required for demonstrable activity, the presence of increased endogenous activity with this concentration of cells rendered the data inconclusive.

Mutual antagonism of methylene blue and sulfathiazole in aerobic respiration.
The Barcroft-Warburg respirometer was used to measure the oxygen consumption of resting pneumococci in the presence of glucose. The cell suspension was previously described except that 0.1 ml of catalase was added for every mg of cells to destroy the hydrogen peroxide formed during respiration. The catalase was prepared from rabbit erythrocytes as described by Sevag and Maiweg (1936). The systems used and the contents of the vessels were as indicated in the tables.

It was found that sulfathiazole (3.8 × 10⁻³ M) inhibited the oxygen consumption of pneumococci in the presence of glucose from 30 to 68 per cent (figure 1, tables 2 and 3). Methylene blue exerted an inhibition of from 20 to 54 per cent. When sulfathiazole and methylene blue were present together, the degree of in-
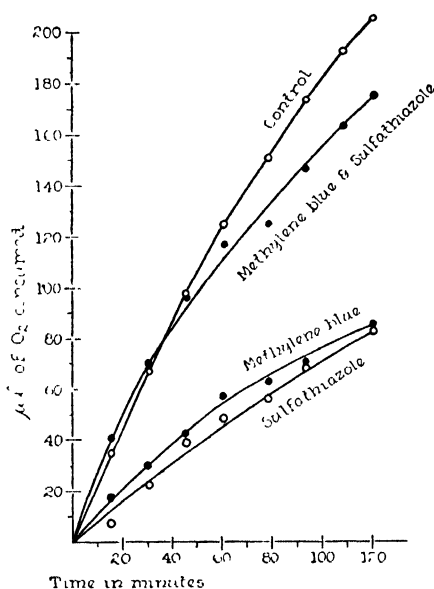
Figure 1. Inhibition of oxygen consumption of pneumococcus, type I, in the presence of glucose by sulfathiazole, methylene blue, and a combination of both.

hibition was greatly decreased, with the Q_{O_2} approaching that of the control. Figure 1 represents a typical experiment. The microliters of oxygen consumed per hour were 125 for the control, 48.5 in the presence of sulfathiazole (61 per cent inhibition), 57.6 in the presence of methylene blue (54 per cent inhibition), and 117 in the combined presence of both (6.4 per cent inhibition). In effect, this represents a mutual antagonism type of phenomena, since the inhibition in the presence of both inhibitors is much less than that in the presence of either alone.

The commercial methylene blue used was 87 per cent pure, with the remaining 13 per cent representing contamination with other dyes such as azure A, azure B, azure C, and methylene violet (Conn, 1946). To determine whether these contaminating dyes might be responsible for the effects observed, the ex-

periments were repeated using azures A, B, and C, and methylene violet. The concentrations of each dye used were based on the total possible concentration of the impurity (13 per cent) that might have been present in methylene blue. This was 0.95 mg per cent final concentration in these systems. Methylene violet could not be tested because of its insolubility in the buffer system. From table 2 it can be seen that all of the azure dyes exert an inhibition of 35 to 57 per cent. The inhibition of the sulfonamide is not relieved by the presence of azure

TABLE 2

Effect of azure dyes on oxygen consumption of pneumococcus, type I, in the presence of glucose, and in the presence and absence of sulfathiazole

SYSTEMS	μl O₂/HR		% INHIBITION	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Control.....	153.8	203.0		
Sulfathiazole..	85.0	133.0	44	35
Azure A.........	92.0	115.0	40	43
Azure A + sulfathiazole.	88.6	106.4	42	48
Control..........	133.0	193		
Sulfathiazole...	72.5	67.5	45	64
Azure B	86.0	125	36	35
Azure B + sulfathiazole....	133.9	114	0	40
Control.....	245.0			
Sulfathiazole.......................	137.5		44	
Azure C.	104.5		57.5	
Azure C + sulfathiazole	152.5		38	

Contents of Warburg vessels:

Controls: 1 mg of pneumococci in 1 ml phosphate buffer (M/20, pH 7.6) containing 0.1 ml catalase; 0.5 ml 6 per cent glucose (M/36, final concentration), 4.2 ml phosphate buffer, 0.3 ml 20 per cent KOH absorbed on filter paper (no. 40) in center well.

Sulfathiazole: Same as controls except 3.7 ml of 150 mg per cent solution of sulfathiazole was substituted for same amount of phosphate buffer (final conc. 3.8×10^{-3} M or approximately 1:1,000).

Dyes: 0.5 ml of 11 mg per cent in phosphate buffer substituted for same amount of phosphate buffer (final conc. approximately 1/100,000).

Gas phase, air; temperature, 37 C; pH 7.6.

A and only slightly by azure C. Azure B was variable in this respect. The results of two experiments are shown ranging from no effect to complete relief of sulfathiazole inhibition. The reason for this inconsistency has not been determined. At any rate, since these dyes are present in these systems in concentrations far greater than could be expected to occur in the commercial preparation of methylene blue, it is unlikely that the effect of the latter is due to the presence of these impurities.

Further evidence to support this was found by comparing the commercial

preparation with a pure methylene blue, recrystallized four times.[2] No significant difference could be demonstrated. It appears, therefore, that these results are a factor of the methylene blue per se. The remaining experiments were conducted with the pure preparation of methylene blue.

In the absence of catalase, though the respiration, due to the accumulated hydrogen peroxide, was reduced, the inhibition by sulfathiazole and methylene blue and their mutual antagonism of inhibition were of the same order as in the presence of catalase. Furthermore, these inhibitors had no effect on the activity of the catalase per se.

Effect of riboflavin on the inhibition of aerobic respiration by sulfathiazole and methylene blue. It was postulated that the mechanism of methylene blue and sulfathiazole antagonism might be due to their affinity for a common site. Since

TABLE 3

Effect of riboflavin on inhibition of oxygen consumption of pneumococcus, type I, in glucose by sulfathiazole and methylene blue

	A. PNEUMOCOCCI				B. PNEUMOCOCCI + RIBOFLAVIN, 20 μg			
Period (min)	Control	Methylene blue	Sulfathiazole	Sulfathiazole + m. blue	Control	M. blue	Sulfathiazole	Sulfathiazole + m. blue
	μl O₂	% inhibition	% inhibition	% inhibition	μl O₂	% inhibition	% inhibition	% inhibition
15	58.3	21	34.5	12.4	56.4	0.3	18.4	−4.5
40	130	17	32.6	6.2	125	4.8	17	−3.1
60	172	21	32.0	9.4	169	12.4	24.2	0.5
90	233	23.6	31.5	10.4	233	18.5	26.4	3.9
120	282	26.3	30.2	9.6	280	19.3	29.3	4.3
150	329	27.0	31.6	11.5	331	23.6	33.0	8.1

Contents of Warburg vessels: Same as in table 2· sulfathiazole, 3.8×10^{-3} M, methylene blue, 0.6 ml 75 mg per cent methylene blue in phosphate buffer (1.67×15^{-4} M).

Organism suspensions: A, Pneumococci in phosphate buffer (M/20 at pH 7.6); B, Pneumococci in phosphate buffer containing 20 μg riboflavin per 1 mg cells. Both suspensions A and B were incubated 2 hours at room temperature before the experiment was begun.

flavoproteins have been implicated as the probable site (Sevag, 1946), the effect of riboflavin was investigated. Riboflavin when added to the reaction system in the Warburg vessels had at times some, but often no, effect on the degree of inhibition of respiration by sulfathiazole. In this variability of the ability of riboflavin to antagonize the sulfonamide inhibition a factor appeared to be exposure of the organisms to riboflavin before the addition of reactants from the side arm. This was definitely confirmed in experiments in which the organisms were previously incubated at room temperature in the presence of riboflavin.

[2] This sample of methylene blue and the samples of azure A, B, C, and methylene violet were kindly supplied by Dr. Walter Ralph of the National Aniline Division of the Allied Chemical and Dye Corporation, Buffalo, New York.

It can be seen from table 3 that the suspension that was incubated in the presence of riboflavin was less inhibited by both sulfathiazole and methylene blue, as well as by the combination of the two. This antagonism, however, is not lasting in that it is eventually overcome by sulfathiazole and the degree of inhibition approaches that which is found in the absence of preincubation with riboflavin. The degrees of inhibition produced by sulfathiazole, methylene blue, and the combination of the two in the untreated suspension are constant and stable over the period of observation. However, with the riboflavin-treated cells, after 15 minutes the inhibition by sulfathiazole is less, 18.4 per cent as compared with 34.5 per cent in the absence of preincubation with riboflavin; methylene blue shows no inhibition (0.3 per cent) and the combination of both is stimulatory (−4 per cent). The inhibitions of these same systems after 150 minutes are almost the same as those of the untreated cells.

Another possibility that may be considered is the occurrence of a chemical reaction between methylene blue and sulfathiazole, with the formation of an inactive complex. In growth systems, however, this type of mutual antagonism does not occur. In fact, it was found that sulfathiazole and methylene blue are synergistic in the inhibition of growth of the pneumococcus. Thatcher (1945) has reported similar synergistic effects with *Escherichia coli*. If a chemical complex were formed, it would have to be one that was inactive in inhibiting respiration and even more active than the individual components in inhibiting growth—a situation difficult to conceive.

The mutual antagonism of sulfathiazole and methylene blue observed in the systems with resting cells involves isolated and limited numbers of reactions mediated by oxidative enzymes. In contrast, the synergistic effects of the two agents observed during growth involve the interactions of a great many enzyme systems, such as various dehydrogenases mediating glucose metabolism, the tricarboxylic acid cycle, and the metabolism of amino acids. The affinities of these systems for methylene blue and sulfathiazole could be assumed to be far greater than the reaction responsible for mutual antagonism between the two agents in systems involving resting cells.

<div align="center">DISCUSSION</div>

These findings emphasize the necessity of assuring the indifference of the reactants of a system before the results from such systems can be interpreted. The ability of methylene blue to antagonize the inhibition of bacterial respiration by sulfonamides must be considered whenever it is used as a reactant. The reported effects of sulfonamides on dehydrogenase systems must be reconsidered from this viewpoint.

Mellon and Bambas (1937) and MacLeod (1939) reported a lack of inhibition of glucose dehydrogenase activity in pneumococci by sulfonamides. The latter, however, did observe inhibition when glycerol, pyruvate, and lactate were used as substrates. Though a detailed description of the components of the systems are given, the order of addition is not described and therefore the results cannot

be adequately evaluated. Clifton and Loewinger (1943) reported inhibition of aerobic respiration and lack of inhibition of methylene blue reduction by *E. coli*. Their interpretation was that the inhibition occurred after the activation of the substrate and that the point of attack must occur between the dehydrogenase and the final hydrogen acceptor. On the basis of the findings presented here, this interpretation may need to be modified. They also reported that methylene blue reduction could not be inhibited even after 2 to 5 hours of exposure of the organism to the drug. It is not stated whether this occurred in the absence or presence of methylene blue. Bucca (1943) reported inhibition of lactic, but not glyceric, dehydrogenase activity of gonococci that had been previously exposed to sulfanilamide in the absence of methylene blue. This would be consistent with our findings. Dorfman and Koser (1942) also reported an increased inhibition of aerobic respiration of dysentery organisms that had been exposed to sulfathiazole for 1 hour. Brazda and Rice (1942) could find no inhibition of D-alanine dehydrogenase by sulfanilamide, measured manometrically. Fox (1942) in an abstract lacking experimental data stated that the lactic dehydrogenase of *E. coli* is not affected by sulfonamides.

Since Altman (1946) found glucose-6-dehydrogenase to be inhibited by sulfanilamide as determined by the aerobic decolorization of 2,6-dichlorophenol indolphenol, it would be advisable to extend this study to other indicators.

In systems containing cells with pretreated riboflavin, sulfathiazole fails to inhibit the respiration at the beginning. However, the system shows gradual emergence of an inhibitory effect. At the end of a 3-hour period there is as much inhibition as in the systems without riboflavin. Sulfathiazole, thus overcoming riboflavin antagonism, appears to have a greater affinity for the sites occupied by riboflavin or its derivatives. In such cases, therefore, the inability of a nutrilite to antagonize an inhibitor would not preclude its being competitively involved. This would explain the inability of riboflavin to antagonize the inhibition of pneumococcal growth by sulfonamides, as previously reported. This would also apply to methylene blue, which as an inhibitor competes with riboflavin for and displaces it from the enzyme site.

SUMMARY AND CONCLUSIONS

The inhibitory effects of sulfathiazole on the glucose dehydrogenase activity and respiration of pneumococcus in the presence of methylene blue, azure dyes, and riboflavin have been studied.

The dehydrogenase activity is inhibited if the exposure of pneumococci to sulfathiazole precedes the addition of methylene blue.

Sulfathiazole, methylene blue, and azure A, B, and C individually exercise inhibitory effects on the respiration of pneumococci. Sulfathiazole and methylene blue show mutual antagonism resulting in the abolition of inhibition by either agent. Azure dyes, with the possible exception of azure B, fail to show a similar mutual antagonism with sulfathiazole.

Riboflavin antagonizes the inhibitions by either sulfathiazole or methylene

blue. The combined presence of methylene blue and riboflavin produces a more effective antagonism to sulfathiazole.

Sulfathiazole or methylene blue is interpreted as displacing riboflavin from the site as the reaction proceeds. Thus, sulfathiazole or methylene blue exercises greater affinity than riboflavin for the enzyme with which the added riboflavin combines.

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STUDIES ON A NEUROSPORA MUTANT REQUIRING UNSATURATED FATTY ACIDS FOR GROWTH[1]

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Although many *Neurospora* mutants requiring water-soluble growth factors have been found (Beadle, 1945; Horowitz *et al.*, 1945; Tatum, 1946), there have been no cases reported of mutants requiring lipid-soluble factors. This was thought strange in view of the discovery of fatty acid requirements for various microorganisms (Hutner, 1942; Hutchings and Boggiano, 1947; Williams and Fieger, 1947; Williams, Broquist, and Snell, 1947). It occurred to the authors that such mutants might have been overlooked by the usual techniques of isolation of *Neurospora* mutants (Beadle and Tatum, 1945). A new selection technique was employed with the view of specifically isolating mutants having fatty acids as growth requirements. With such a technique a mutant requiring unsaturated fatty acids has been obtained and its growth characteristics have been studied.

METHODS

Isolation of the mutant. The selection technique used to obtain the mutant was that developed by Lein, Mitchell, and Houlahan (1948). The method consists essentially of picking probable mutants from mixtures of wild type and mutant ascospores germinating on minimal medium. The mutants are readily distinguishable and are placed in minimal medium supplemented with the appropriate growth factor. The cultures that grow are tested to determine whether this growth factor is a necessary requirement.

The details of the procedures used that yielded the mutant are as follows: Conidia from 10 test tube cultures of *Neurospora crassa* (strain 7A, isolated from a cross between 5256A and 5297a) were suspended in distilled water and rayed with a "sterilamp" for a time sufficient to kill 90 to 95 per cent of the conidia. The rayed conidia were added to cultures of the opposite mating type (strain 8a, also isolated from a cross between 5256A and 5297a) that had been growing for 5 days in 20 petri plates on a medium developed by Westergaard and Mitchell (1947). Profuse perithecia were formed and mutants were isolated by the method cited above. A total of 1,201 probable mutants were picked over a 10-day period and placed on a medium consisting of minimal medium (Beadle and Tatum, 1945) supplemented with 500 mg per cent of linoleic acid added in emulsion form. Of the number picked, 201 yielded cultures, and these were tested for a fatty acid requirement. One of these cultures had such a requirement and was designated S-11. The culture was crossed to a wild type of the opposite mating type. Ascospores were isolated in order from 10 asci and tested to determine

which had lipid requirements. Typical Mendelian segregation occurred, so it was established that S-11 was a true mutant.

Materials and procedures. The unsaturated fatty acids used in determining the growth requirements were obtained from the Hormel Foundation and were of very high purity. The iodine number (Wijs) of the oleic acid was 89.56 (theoretical value 89.87), that of the linoleic acid was 180.70 (theoretical value 181.03), and that of the linolenic acid was 272.5 (theoretical value 273.51). The saturated fatty acids that were used were obtained from the Eastman Kodak Company.

The fatty acids were added in emulsion form. One per cent emulsions were prepared with a Waring blender, and these were diluted to yield the desired concentrations. The solid compounds were first melted before being emulsified. All emulsions were prepared on the day they were used.

For the growth studies, one drop of a conidial suspension was used for inoculation. This was added to 125-ml flasks containing 20 ml of minimal medium supplemented with the growth factor. The flasks had been previously sterilized by autoclaving at 15 pounds for 10 minutes. The inoculated flasks were incubated at 25 C for 96 hours; the mycelia were removed, pressed out, dried at 100 C, and weighed.

RESULTS

The mutant S-11 failed to grow in minimal medium or minimal medium supplemented with 1 ml of 1 per cent lauric acid, myristic acid, palmitic acid, or stearic acid. The mutant did grow when minimal medium was supplemented with oleic acid. The mutant also grew in low concentrations of linoleic acid and linolenic acid, but grew slightly or not at all at higher concentrations of these fatty acids. In view of the demonstrated inhibitory effect of unsaturated fatty acids on bacteria (Kodicek and Worden, 1945) it was thought that inhibition might be playing an important role. Polyoxyethylene sorbitan monostearate ("tween 60") was used in an attempt to relieve this inhibition, since Williams and Fieger (1947) reported that certain surface tension depressants render oleic acid nontoxic to lactic acid bacteria.

The results of growth experiments using various concentrations of fatty acids with and without two different concentrations of "tween 60" are presented in figure 1. The lower portion of the figure represents the growth obtained by various amounts of the three fatty acids in the absence of "tween 60." The growth mixture consisted of 20 ml of minimal medium, 1 ml of the concentration of fatty acid listed on the abcissa, and 1 ml of distilled water. The graph shows vividly the inhibition by the higher concentrations of linoleic and linolenic acids. The 1 per cent emulsion of linolenic acid completely inhibited growth. The results of experiments in which 1 ml of 1 per cent "tween 60" was added in place of the distilled water are presented in the middle portion of figure 1. Comparison with the experiment using no "tween 60" shows that there is a marked increase in growth at the higher fatty acid concentrations. This is in all probability due to partial relief of the inhibition caused by these compounds. The upper portion of figure 1 contains the growth curves obtained when 2 per cent "tween 60" is

added. The curves of the fatty acids indicate further relief from the inhibitory effects of these compounds.
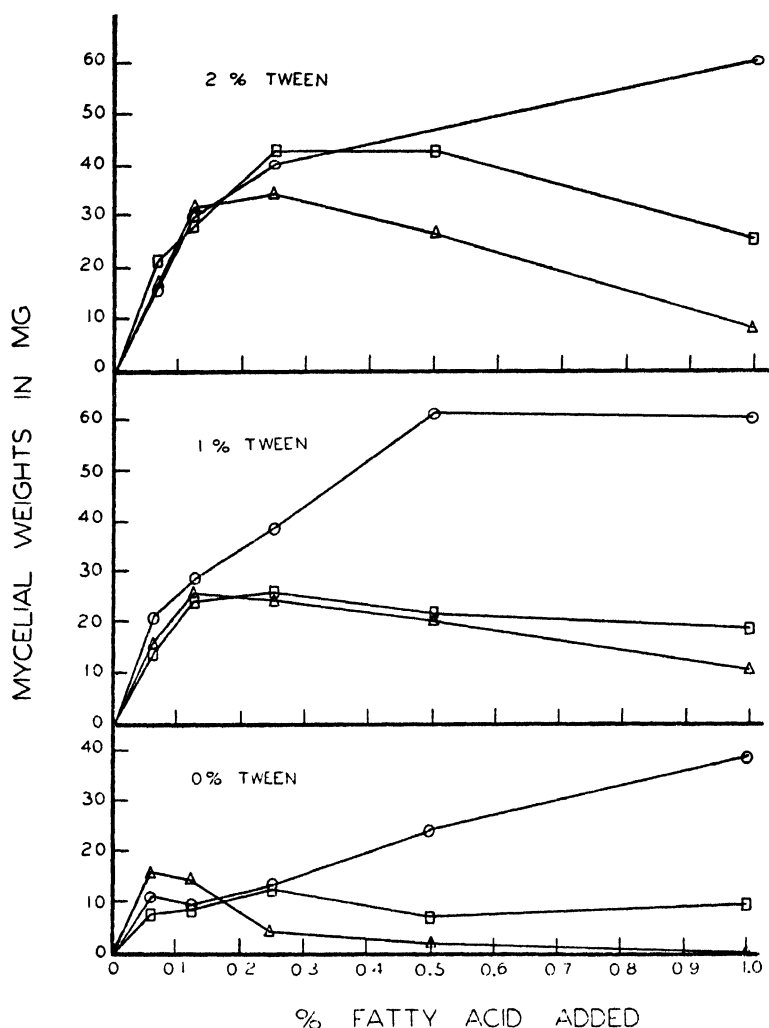
Figure 1. Growth of S-11 mutant in minimal medium supplemented with various concentrations of fatty acids and 0, 1, and 2 per cent "tween 60." Circles represent oleic acid values; squares, linoleic acid; and triangles, linolenic acid.

DISCUSSION

In the case of mutant S-11 we have an example of a substance that enables the mutant to grow and also acts as an inhibitor of its growth. It is improbable that these two effects on the organism are related since they can be dissociated through the use of polyoxyethylene sorbitan monostearate. The results also indicate that the degree of inhibition by the unsaturated fatty acids depends on

the number of unsaturated bonds. Thus, judging from the extent of growth one obtains at the higher concentrations of the fatty acids in the absence of the surface tension depressant, the oleic acid is less inhibitory than linoleic acid and linoleic acid is less inhibitory than linolenic acid. The mechanism of this inhibition and its relief by a synthetic detergent is as yet unknown.

The fact that S-11 will grow when it is given oleic acid, linoleic acid, or linolenic acid indicates that the syntheses of these three compounds are related. The simplest scheme of manufacture of these compounds would be the synthesis of linoleic acid from oleic acid and linolenic acid from linoleic acid by dehydrogenation. This view has for support the finding in the rat that linoleic acid and linolenic acid but not oleic acid will prevent nutritional disorders produced by diets devoid of fats (Burr *et al.*, 1929, 1930, 1932). Evidently the rat can make its oleic acid but not linoleic or linolenic acids. This is further supported by the isotope work of Bernhard and Schoenheimer (1940). The rat, therefore, seems to have a metabolic block between oleic acid and linoleic acid, but in the *Neurospora* mutant studied the block lies before oleic acid. This is represented in the following scheme:

$$\xrightarrow{\text{S-11}} \text{oleic acid} \xrightarrow{\text{Rat}} \text{linoleic acid} \longrightarrow \text{linolenic acid}$$
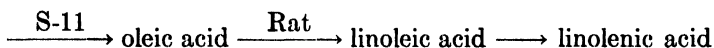
Though the growth experiments indicate that unsaturated fatty acids play a necessary role in some cellular process, the results do not enable us to determine what this role is. The work of Axelrod *et al.* (1947), Williams and Fieger (1947), and Williams, Broquist, and Snell (1947) indicates that there is a relation between biotin and oleic acid since oleic acid can replace the biotin requirement in certain bacteria. Since the minimal medium for *Neurospora* contains biotin, the mutant studied must have some need for an unsaturated fatty acid other than that related to its biotin requirement.

SUMMARY

A *Neurospora* mutant has been isolated which requires for growth either oleic acid, linoleic acid, or linolenic acid. It does not grow when supplied with saturated fatty acids. The unsaturated fatty acids that make growth possible have an inhibitory effect on growth, the amount of inhibition varying directly with the degree of unsaturation of the fatty acids. This inhibition is relieved by the synthetic detergent polyoxyethylene sorbitan monostearate. The growth studies are consistent with the view that linoleic acid is synthesized from oleic acid and that linolenic acid is synthesized from linoleic acid. In the mutant studied, the metabolic block lies before the synthesis of oleic acid.

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THE STREPTOCOCCUS LACTIS HOST-VIRUS SYSTEM[1]

I. Factors Influencing Quantitative Measurement of the Virus

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The host-virus system used in this study was selected for two reasons. First, it seemed desirable to apply to other systems some of the newer techniques used so successfully in the study of the T series of viruses for *Escherichia coli* (Delbrück, 1946; Anderson, 1946; Cohen, 1949). Second, recognition of virus infection of starter cultures used in the dairy industry for the manufacture of cheese, butter, and sour cream has become widespread (Nelson *et al.*, 1939; Johns and Katznelson, 1941; Whitehead and Hunter, 1945; Mattick *et al.*, 1944).

Most of the publications dealing with viruses attacking *Streptococcus lactis* and *Streptococcus cremoris* are based on work done by those who were primarily interested in the control of these agents. Few workers have studied these host-virus systems from the standpoint of the intrinsic nature of the relationship of virus to host. Viruses for *S. lactis* and *S. cremoris* have previously been assayed by activity tests in milk (Nelson *et al.*, 1939; Hunter, 1943; Nichols and Wolf, 1944), by lysis in broth (Nelson *et al.*, 1939; Johns and Katznelson, 1941; Nichols and Wolf, 1945), and by the counting of plaques (Whitehead and Cox, 1936; Nelson *et al.*, 1939; Johns and Katznelson, 1941; Hunter, 1943; Wolf *et al.*, 1946; Nelson and Parmelee, 1949). The heat resistance of the viruses attacking the bacteria of cheese starter cultures was studied by Nichols and Wolf (1945). Nelson *et al.* (1939) observed that the composition and the pH of the medium influenced the lysis of *S. lactis* by homologous virus. Whitehead and Hunter (1939) reported that the detrimental effects of virus on cheese starter organisms could be largely overcome by increasing the size of the inoculum of the starter culture. The following report is concerned with the development of a reliable method of estimating virus by a plaque count assay and with a study of some of the factors influencing the accuracy of this method.

EXPERIMENTAL METHODS AND RESULTS

Host-virus system. The host-virus system used in this study was strain 122-4 of *S. lactis* and its homologous virus, which were obtained from Dr. F. J. Babel of Iowa State College. The virus was purified by three successive plaque isola-

tions after it was received. The morphology of viruses for *S. lactis* and *S. cremoris* as studied by electron microscopy was reported by Parmelee *et al.* (1949) and independently by Cherry (1949). Figure 1 shows a field of particles of 122-4 virus shadowed with gold. The average dimensions of these particles are as follows: diameter of head structure 73 mμ, length of tail 160 mμ, width of tail 20 mμ, and over-all length 230 mμ. The nature of the tangled mass of filaments with embedded virus particles is unknown.

The bacteria were grown in a medium consisting of 1 per cent Difco tryptone, 0.2 per cent glucose, and 0.3 per cent Difco yeast extract adjusted to pH 7.0. A buffered medium could not be used for reasons that will be reported in the sec-
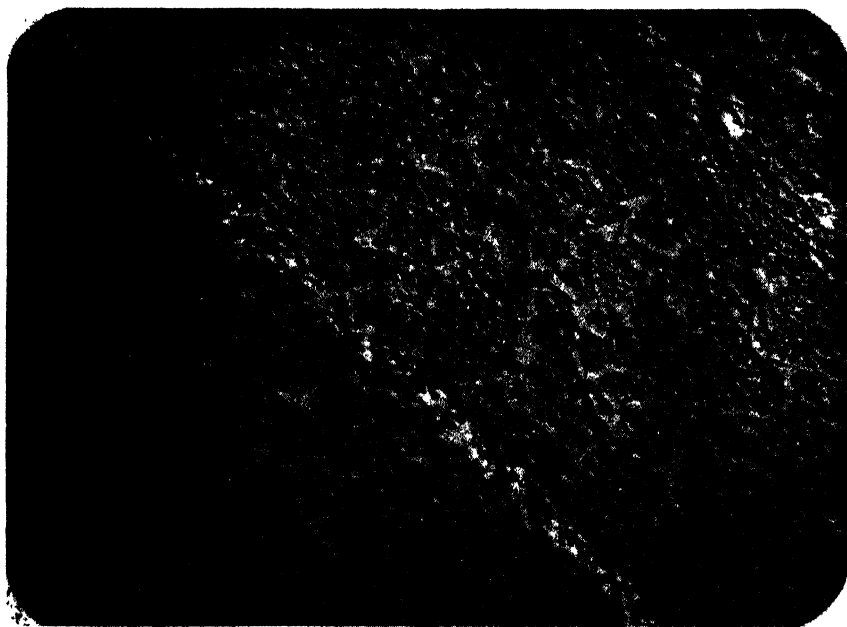
Figure 1. S. lactis 122-4 virus. Shadowed with gold. $\times$ 21,000. Note granular appearance of virus and mass of taillike filaments at one corner of the picture.

ond paper of this series. Therefore, cells for a single experiment were prepared by adding a 10 per cent inoculum of a 14- to 18-hour broth culture to fresh medium and incubating the culture at 37 C for 3 to 5 hours. The bacteria were collected by centrifugation and resuspended in distilled water to give a concentration of about 10^9 per ml (plate count). These preparations were used for lysis, adsorption, and one-step growth curve measurements, and for making plaque counts.

Stock virus consisted of lysates obtained by adding sufficient virus to give 10^3 to 10^4 plaque-forming particles per ml to medium containing 10^7 cells per ml and incubating at 30 C until lysis had occurred. If the pH dropped too rapidly, aliquots of sterile 0.5 M sodium hydroxide were added to maintain the reaction above 6.0.

Plaque counts. The plaque-counting method was based on the technique of Gratia (1936). It consisted of mixing a small amount (0.5 to 1.0 ml) of a virus dilution in broth with 3.0 ml of 0.7 per cent melted agar medium and a small amount of the suspension of cells. This material was poured on the surface of plates containing 20 ml of solidified 1.5 per cent agar medium and allowed to spread evenly in a thin layer. Thus, the plaques were formed in a thin layer of medium resting on an uninoculated nutrient base. The cell concentration was 2.0 to 4.0 $\times$ 10^7 per ml of mixture plated. Plates were incubated for a minimum of 8 hours at 25 to 30 C before counts were made. A statistical analysis showed that the accuracy of the method was equal to that of the plaque-counting method analyzed by Hershey *et al.* (1943) for the assay of virus of *E. coli*.
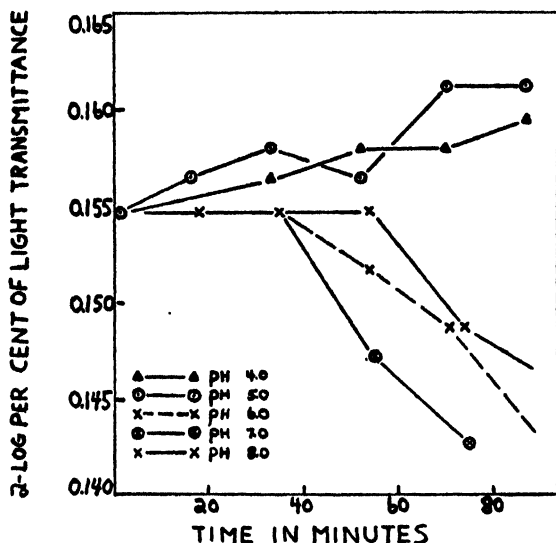
Figure 2. The effect of pH of the medium upon lysis of *S. lactis* 122–4 by virus at 30 C.

Effect of the pH of the medium on the lysis of cells by virus. Figure 2 illustrates the result of infecting cells of *S. lactis* 122-4 with virus in media adjusted to pH 4.0, 5.0, 6.0, 7.0, and 8.0. At pH 4.0 the cells either were not infected by virus or infection did not prevent some cellular multiplication. At pH 5.0 some cells were probably infected since incomplete lysis occurred at the end of the normal latent period of virus growth (about 35 minutes). The cells were completely lysed at pH 6.0, 7.0, and 8.0, but at the highest pH the latent period of virus growth was extended by about 10 minutes. It appears that pH 5.0 is near a critical level below which lysis does not occur.

Effect of the pH of the medium on the adsorption of virus. In the foregoing experiment the residual virus was determined after exposure of *S. lactis* cells to virus for 10 minutes at 30 C in the broth medium adjusted to various pH levels. The virus to cell ratio was about 4. The cells were separated by centrifugation at the end of the adsorption period, and the free virus was assayed by the plaque-

counting technique. In table 1 the results are summarized. It is apparent that virus adsorption fell off somewhat on each side of neutrality, but on the alkaline side the decrease was slow. The high figures at pH 4.0 and 5.0 probably represent rapid virus inactivation since this result would manifest itself as an apparent increase in adsorption.

Effect of the inoculum on the number and size of plaques. The size of the inoculum was an important factor in determining the size and number of plaques

TABLE 1

Adsorption of virus by S. lactis 122-4 at various pH levels

INITIAL pH OF MEDIUM	PLAQUE COUNT OF FREE VIRUS—DUPLICATE PLATES	PLAQUE COUNT OF FREE VIRUS PER ML OF ADSORPTION TUBE $\times 10^7$	PER CENT OF INITIAL VIRUS* ADSORBED†	MACROSCOPIC LYSIS
4	346–307	6.5	57	—
5	362–332	6.9	54	—
6	436–436	8.7	42	+
7‡	165–173	3.4	77	+
7‡	187–191	3.8	75	+
8	247–276	5.2	65	+

* Initial virus per ml of adsorption tube, 1.5×10^8.
† Calculated by difference.
‡ Separate determinations.

TABLE 2

Effect of varying the size of the inoculum of S. lactis 122-4 on the size and number of plaques produced

CELL COUNT PER ML OF MATERIAL PLATED, $\times 10^6$	PLAQUE COUNT DUPLICATE PLATES	PLAQUE COUNT PER ML OF VIRUS DILUTION	MEAN PLAQUE SIZE
			mm
3.2	164–188	362	2.1
6.4	202–189	391	2.1
9.6	215–213	428	1.9
13.0	255–213	468	1.7
19.0	249–266	515	1.6
26.0	245–245	490	1.3
39.0	236–234	470	1.2
52.0	259–260	519	1.1
65.0	245–209	454	0.9

formed. This is illustrated by the data of table 2 in which plaque size was shown to decrease as the number of cells in the plating mixture increased. The number of plaques formed decreased rapidly as the cell concentration fell below 1.3×10^6 per ml. However, it is apparent that there is a fourfold range of cell concentration (1.3 to 5.2×10^7 per ml) over which little variation occurs in the number of plaques formed. Variations in plaque size were largely accounted for by nonuniformity of bacterial growth.

Proportionality of plaque count to virus concentration. A plaque count assay must measure virus in proportion to its concentration in successive dilutions.

TABLE 3

Proportionality of the concentration of S. lactis 122-4 virus to the plaque count

DILUTION	LOG DILUTION	PLAQUE COUNT DUPLI-CATE PLATES	PLAQUE COUNT/ML DILUTION	LOG PLAQUE COUNT/ML DILUTION
2	0.30	444–407	851	2.93
2.5	0.40	369–372	741	2.87
3.0	0.48	275–295	570	2.76
3.5	0.54	243–257	500	2.70
4.0	0.60	252–235	487	2.69
5.0	0.70	185–222	407	2.61
6.0	0.78	160–158	318	2.50
10.0	1.00	83–100	183	2.26
20.0	1.30	44–44	88	1.94
40.0	1.60	24–18	42	1.62
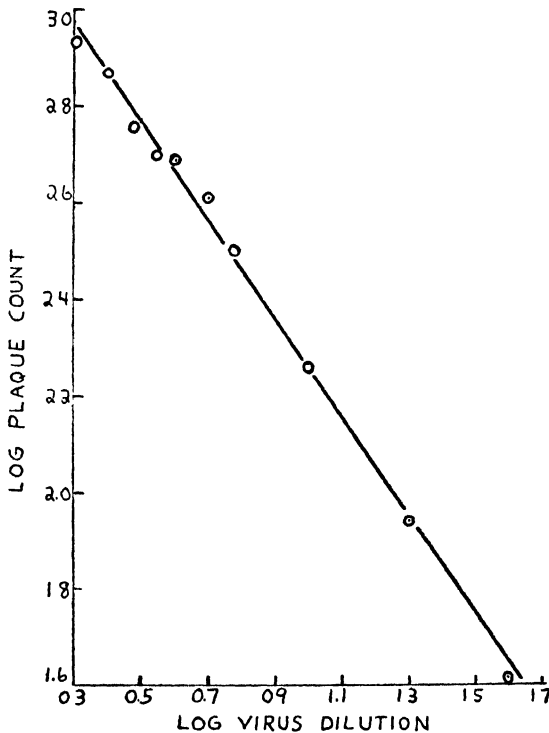
Figure 3. The proportionality of the concêntration of virus for *S. lactis* 122-4 to the plaque count.

Stock virus was diluted in broth to contain about 1,700 plaque-forming particles per ml. Additional dilutions were made as shown in table 3. Then, 0.5 ml of

each of the 10 dilutions were plated in duplicate in the manner previously described. Figure 3 shows the result of plotting the logarithms of the dilutions against the logarithms of the plaque count per ml of the dilution. There is a strict proportionality of virus concentration to plaque count for a 20-fold range of virus dilution.

Efficiency of the plaque count method. The efficiency of the plaque count was determined by obtaining the best measure of the actual number of virus particles in a dilution of virus and comparing this value with a plaque count obtained on the same dilution. Ten tubes of each of four consecutive 10-fold virus dilutions in broth were inoculated with *S. lactis* cells to give a concentration of 5×10^6 cells per ml. These tubes were incubated at 30 C for 12 hours. At this time the tubes were either completely lysed or had a heavy growth of bacteria.
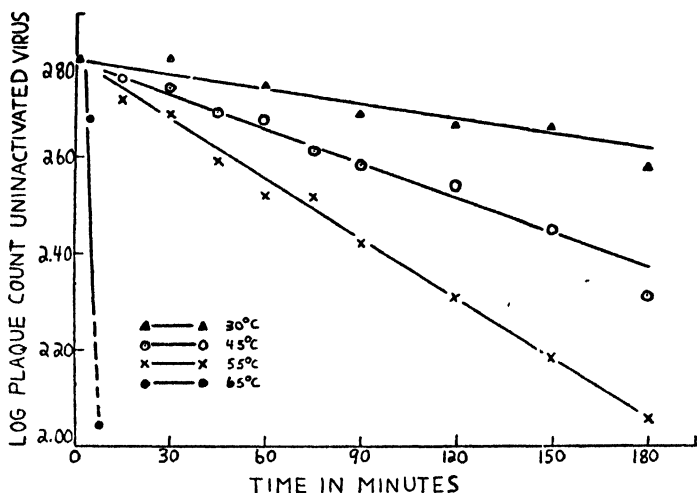
Figure 4. The log of uninactivated virus for *S. lactis* 122-4 in broth at pH 6.8 at various temperatures as a function of time.

The number of tubes of each dilution in which lysis occurred was noted. The dilutions of virus that were used were 1×10^{-9}, 10^{-10}, 10^{-11}, and 10^{-12}. By use of the code, 10-9-0, for the number of tubes that were lysed in the three higher dilutions and by reference to the tables of Halvorson and Ziegler (1933), the most probable number of virus particles in the 10^{-11} dilution was found to be 1.7 per ml. The range of the estimate obtained by the M.P.N. value can be determined by applying the statistic for log range (Eisenhart and Wilson, 1943):

Log range $= \log \lambda \pm (1.96)(0.166)$

$\lambda =$ maximum likelihood estimate from the Halvorson-Ziegler tables.

The value obtained was 0.8 to 3.6×10^{11} virus particles per ml of stock virus; this value has a confidence coefficient of 0.95. An average plaque count was obtained from five replicate plates prepared from the 10^{-8} virus dilution tube. The number of plaque-forming particles per ml of stock virus was found to be

2×10^{10}. Thus, the ratio of the virus concentration obtained by the log range estimate to the plaque count estimate measures the efficiency of the latter. This value varies between 0.05 and 0.25.

Effect of temperature on virus inactivation. The rate of inactivation of 122-4 virus in broth was studied at 30, 45, 55, and 65 C for periods of time up to 180

TABLE 4

Specific reaction rates for thermal inactivation of virus for S. lactis 122-4

TEMPERATURE C	$k \times 10^3$	LOG $k \times 10^3$	T ABSOLUTE	$10^3 \times 1/T$ ABSOLUTE
30	2.300	0.362	303	3.30
45	5.359	0.729	318	3.14
55	9.453	0.975	328	3.05
65	263.58	2.421	338	2.96
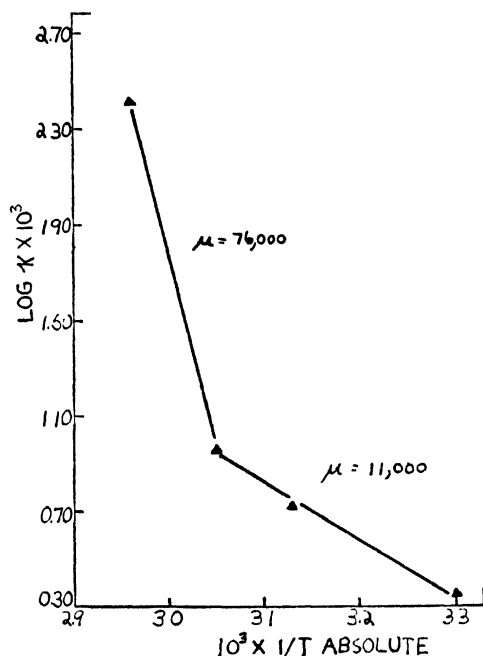
Figure 5. The log of the first-order specific reaction rates for inactivation of virus for *S. lactis* 122-4 as a function of temperature.

minutes. Figure 4 presents the results graphically. The specific reaction rate (k) was calculated at each temperature by using the equation:

$$k = \frac{2.3}{t} \log \frac{\text{final concentration}}{\text{initial concentration}}.$$

The last expression of the equation was obtained by estimating the slope of the curves for inactivation of virus at each temperature (figure 4). The logarithms

of the k values for each temperature were then plotted against the reciprocals of the corresponding absolute temperatures. These data are given in table 4 and figure 5. This is the method that was devised by Arrhenius for demonstrating the relationship between reaction rate and temperature. From this plot the temperature characteristic (μ) of thermal inactivation of the virus was calculated by using the relationship

$$\mu = \text{slope} \times 4.6 \times 10^3.$$

The μ value obtained for the linear part of the curve including the three lower temperatures (30 C, 45 C, and 55 C) was 11,000 gram calories. Between 55 C and 65 C the rate of virus inactivation increased rapidly since most of the plaque-forming capacity was destroyed within 15 minutes. Although determined by only two points, the μ value has risen to about 76,000 gram calories. The rate of virus inactivation below 55 C was slow and indicated that the effect of exposure of the virus to a temperature of 45 C for short periods (1 minute) while making plaque counts was negligible.

DISCUSSION

The data presented here show that the interaction of S. lactis 122-4 and its homologous virus can be studied quantitatively by use of the plaque-counting technique. Assay of the virus by the activity method of Krueger (1930) was investigated and found to be unsatisfactory for this particular system. The most important factors affecting the accuracy of both the activity method and the plaque count are the size and activity of the cell inoculum and the pH of the medium. These can be standardized much more easily in the plaque-counting techniques since the time required for lysis of the bacterial cells is not of primary importance to the results of the virus assay. The uninoculated base of agar medium used in the plates for plaque count assay of virus supplied sufficient control of the pH without the necessity of adding buffer salts. It will be shown in the next paper of this series that many electrolytes prevent the adsorption of virus to the cell. Since S. lactis is a homofermentative organism, it rapidly lowers the pH of an unbuffered medium by the production of lactic acid. At a pH of 5.0 or below the cells are not lysed by the virus, so that plaques must be clearly visible before this critical level is reached.

The efficiency of the plaque count assay for virus active against S. lactis is not high. However, routine assays of stock virus conducted over periods of several weeks showed that consistent results were obtained. The inherent characteristics of the plaque count method (Anderson, 1949) indicate that its efficiency is seldom if ever 100 per cent. Ellis and Delbrück (1939) reported that the probability of plaque formation by a particle of one of their viruses for E. coli was about 0.4.

The effect of very short exposures of 122-4 virus to temperatures below 55 C is negligible. However, the rapid rate of virus inactivation between 55 and 65 C indicates that an important mechanism of the virus particle is being destroyed.

SUMMARY

The morphology of the virus 122-4 active against *Streptococcus lactis* was found to agree closely with that reported for other viruses of *S. lactis*.

The pH of the medium was found to be a controlling factor in lysis of the bacteria by virus. At pH values below 5.0 lysis did not occur. At pH 8.0 lysis was delayed. Virus adsorption was highest at pH 7.0. The plaque count technique gave a reliable measure of virus concentration if the size of the inoculum was kept in the range 1 to 5×10^7 cells per ml of mixture plated.

A strict proportionality existed between virus concentration and plaque count for a 20-fold range of dilution.

The effect of temperature on the inactivation of virus at 30, 45, 55, and 65 C was studied.

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THE STREPTOCOCCUS LACTIS HOST-VIRUS SYSTEM[1]

II. CHARACTERISTICS OF VIRUS GROWTH AND THE EFFECT OF ELECTROLYTES ON VIRUS ADSORPTION

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The development of a technique for isolating one step in the growth of a virus for *Escherichia coli* was described by Ellis and Delbrück (1939). This method permits the determination of the latent period for virus growth and the yield of virus per bacterium under defined physiological conditions. If the composition of the medium is varied, the resulting effect on virus growth will be reflected in changes in the characteristics of the one-step growth curves. It seemed desirable to apply the one-step growth technique to a study of the characteristics of virus growth in the *Streptococcus lactis* host-virus system.

In attempting to study virus production by this system in a synthetic medium, difficulty was experienced in infecting the cells with virus. This was discovered to result from the presence of buffer salts in the medium. Therefore, a study of the effect of electrolytes on virus adsorption was undertaken.

There are many reports of the effect of electrolytes on virus multiplication. Scribner and Krueger (1937) have reviewed the work reported up to 1937. Relatively few workers have analyzed the influence of salts on adsorption of virus by the host cells. Krueger and Strietmann (1938) observed that in the presence of M/8 sodium sulfate the adsorption of virus by a culture of *Staphylococcus aureus* was reduced by a factor of 2. Scribner and Krueger (1937) reported that at 0 C there was no significant difference in the quantity of virus adsorbed by *S. aureus* cells between those suspended in 0.25 M sodium chloride and those suspended in a medium without salt. Gest (1943) concluded that 0.01 M magnesium chloride had no observable effect on the adsorption of virus by *E. coli* cells. Hershey *et al.* (1943) noted that a relatively high concentration of sodium and a low concentration of calcium ions were essential for plaque formation for T$_2$ virus. Part of the present report is concerned with the determination of optimal salt concentrations for adsorption of virus on *S. lactis* host cells.

MATERIALS AND METHODS

The host-virus system used consisted of a strain of *S. lactis* 122-4 and its homologous virus. Both virus lysates and cell suspensions were prepared as de-

scribed in a previous paper (Cherry and Watson, 1949). The bacteria for all experiments were grown in an unbuffered medium consisting of 1 per cent tryptone (Difco), 0.3 per cent yeast extract (Difco), and 0.2 per cent glucose. This medium will be referred to as "complete," and a similar medium from which tryptone was omitted will be called "deficient." The initial pH of the media for all experiments was 7.0. Adsorption mixtures of cells and virus were incubated in the water bath at 30 C for 10 minutes. Aliquots of 1 ml of the mixtures were removed and centrifuged for 6 minutes at 2,400 rpm in the angle centrifuge to sediment the cells. Free virus in the supernatants was assayed by the plaque count method previously described (Cherry and Watson, 1949). Adsorbed virus was given by the difference between the initial concentration and the free virus at the end of the adsorption period. Salt solutions were prepared in either 0.1 M or 0.2 M concentrations in sterile distilled water and stored at 1 C. The pH was adjusted to 7.0 with 0.5 N sodium hydroxide when necessary. The salts were added to the medium immediately before beginning an experiment. The effect of electrolytes on lysis of cells by virus was determined by making turbidity readings after 2 to 4 hours of incubation at 30 C. The virus to cell ratio in all experiments was about 4, and the cell concentration was about 5×10^7 per ml.

One-step growth experiments were conducted according to the technique of Delbrück and Luria (1942). After an adsorption period of 10 minutes at 30 C, free virus was determined as described above. At the same time adsorption was arrested by diluting the cell-virus mixture to 10^{-5} or 10^{-6} and continuing the incubation. Aliquots were removed periodically and after suitable dilution were assayed for total virus by the plaque-counting method. All dilutions were made in broth of the same composition as that used in the adsorption tube. The average burst size was calculated from the formula given by Delbrück and Luria (1942). Bacterial cell counts were computed by arbitrarily multiplying the plate count by 2, since it was found that this figure expressed the ratio of the count obtained by the microscopic method to that given by the plate count.

RESULTS

The effects of electrolytes on lysis of cells by virus. The influence of several salts on the lysis of *S. lactis* 122-4 by virus was tested. It was found that the omission of tryptone from the medium reduced virus adsorption from about 80 per cent to 20 to 30 per cent of the initial virus. Therefore, a medium free of tryptone was considered "deficient" for virus adsorption. The stimulating effect of electrolytes could be measured by noting the increase in adsorption resulting from the addition of salts. "Deficient" medium supplemented with salts never gave better adsorption than could be obtained in the same medium to which 1 per cent of tryptone was added ("complete"). Therefore, virus adsorption in the "complete" medium was determined in each experiment and served as a marker of adsorption efficiency. The salt concentrations employed ranged from 0.001 M to 0.1 M. The results of these experiments are presented in figures 1, 2, and 3, in which the percentage of light transmittance after a 2- to 4-hour incubation

period is plotted against molar concentrations of the salts added. The peaks of the curves represent lysis of cells at the corresponding salt concentrations. Lysis usually began at the end of the latent period of virus growth (32 minutes). Sodium and potassium phosphate and magnesium sulfate were most effective at 0.005 to 0.01 M concentrations, whereas calcium chloride was effective over a larger range (0.005 to 0.05 M). Sodium and potassium sulfate had their greatest activity at 0.01 M concentrations, sodium chloride at 0.02 to 0.05 M levels, and potassium chloride at the 0.02 M level. Lithium chloride (figure 3) was somewhat effective at 0.05 to 0.1 M concentrations and sodium acetate at 0.02 to 0.05 M
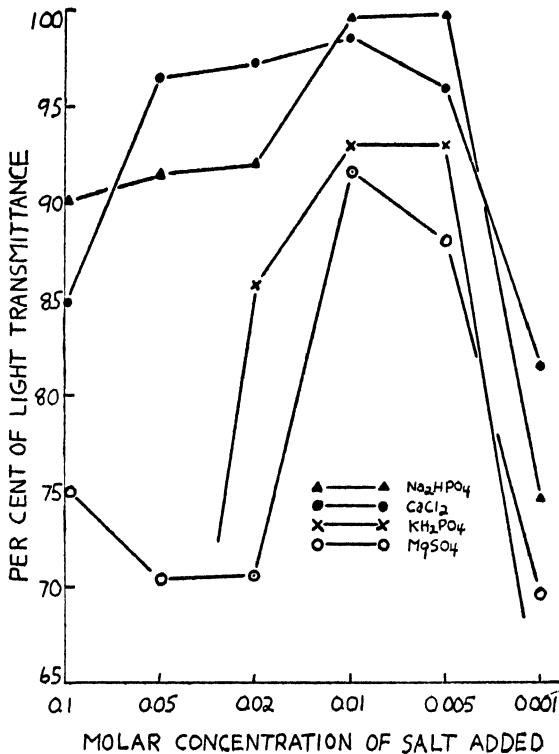
Figure 1. Effect of salts on lysis of *S. lactis* 122–4 by virus at pH 7.0 and 30 C.

salt levels. Manganous sulfate was toxic to the growth of bacteria at all levels, and cells were not lysed by virus in the presence of the salt. Potassium oxalate in a 0.1 M concentration inhibited growth of the cells and allowed no appreciable lysis even at lower salt levels. Sodium citrate was toxic at the higher concentrations and appeared to be somewhat inhibitory to the bacteria at all salt levels.

These data indicate that many electrolytes are active in promoting lysis of *S. lactis* cells by virus. The effective range of salt concentrations is usually rather small and in some cases very sharp.

The effect of electrolytes on virus adsorption. Experiments were performed in order to test whether or not the effects noted above resulted from stimulation of

virus adsorption. The salt concentration giving the greatest activity for cellular lysis was found to be the one giving the greatest adsorption of virus to the cell as measured by the plaque count assay. Table 1 shows the results obtained with potassium phosphate. As predicted from figure 1, maximum stimulation was obtained in the presence of 0.01 M phosphate. Lower or higher salt concentrations depressed the adsorption of virus below that obtained in the "deficient" medium without added phosphate. The effect of calcium chloride is evident in the data of table 2, in which the greatest activity was obtained in the presence of 0.01 and 0.02 M salt concentrations. Cellular lysis was noted only at these salt levels. Similar results were obtained with sodium citrate (table 3), with the ex-
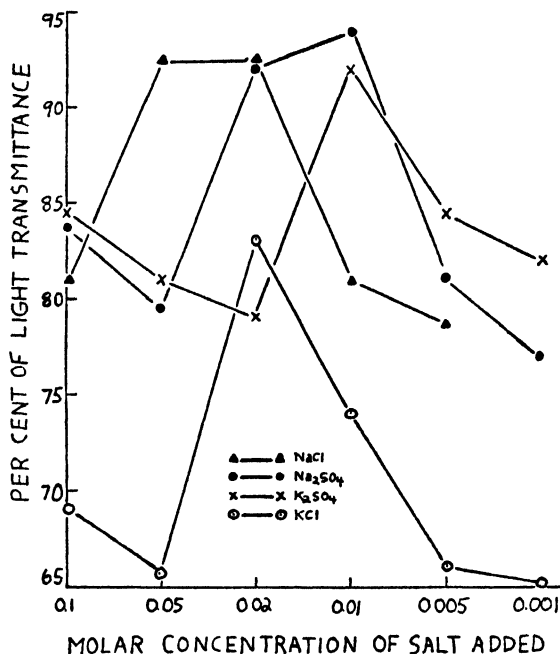
Figure 2. Effect of salts on lysis of S. lactis 122-4 by virus at pH 7.0 and 30 C.

ception that cellular lysis was not observed at any level of salt concentration used. Potassium and sodium chloride, magnesium sulfate, and sodium acetate were tested in the same way and found to stimulate or depress virus adsorption in accordance with the previously noted effect on cellular lysis.

An experiment was performed to show what part of the total adsorbing ability of the "complete" medium was contributed by yeast extract and what by tryptone. In the medium composed of yeast extract and glucose, 21 per cent of the initial virus was adsorbed in 10 minutes at 30 C. The tryptone plus glucose medium supported 59 per cent adsorption under the same conditions. The sum of these (80 per cent) equals the adsorption given by the "complete" medium (82 per cent). If the concentration of yeast extract in the "deficient" medium was increased from 0.3 per cent to 1.0 per cent, virus adsorption was approxi-

mately as good as in the "complete" medium. This fact indicates that the inability of the "deficient" medium to support virus adsorption is the result of quantitative rather than qualitative factors.

One-step growth experiments in "complete" medium. Typical one-step growth curves of virus on *S. lactis* 122–4 in "complete" medium at 30 C and an initial pH of 7.0 are shown in figure 4. In table 4, experiments 1 and 3, these data are summarized. The latent period of virus growth is in the range of 31 to 33 minutes, and the rise period is 15 to 18 minutes when the multiplicity of infection is about 3. With virus to cell ratios of less than 1, similar results were obtained.
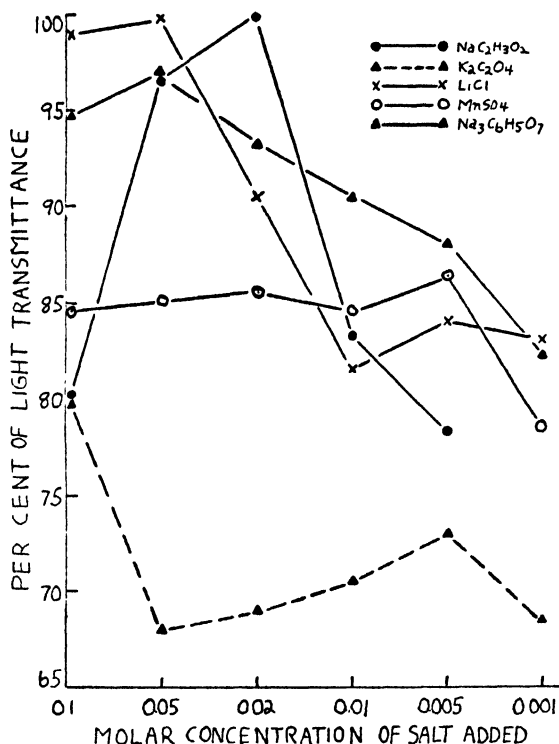
Figure 3. Effect of salts on lysis of *S. lactis* 122–4 by virus at pH 7.0 and 30 C.

The average yield per infected bacterial cell was about 70 plaque-forming particles of virus. Final concentrations of virus were about 20 times the initial input.

One-step growth experiments in "deficient" medium. Experiment 4 of figure 5 illustrates the effect of removing tryptone from the "complete" medium. The medium was not "deficient" for virus adsorption since it was supplemented by the addition of monobasic potassium phosphate to give a 0.01 M concentration. This results in adsorption of virus equivalent to that obtained in "complete" medium. However, the latent period of virus growth is increased by about 20 minutes and the average burst size is reduced to 24 (table 4). The final to initial

TABLE 1

The effect of potassium phosphate on the adsorption of virus by S. lactis 122-4

MEDIUM	PLAQUE COUNTS OF FREE VIRUS DUPLICATE PLATES	VIRUS ADSORBED PER ML × 10⁷	PERCENTAGE OF INITIAL VIRUS* ADSORBED	EXPECTED PERCENTAGE OF ADSORPTION†
"Complete"‡	174–175	14.5	80	75–85
"Deficient"§	57–66	6.0	33	20–30
"Deficient" + 0.001 M KH₂PO₄	71–79	3.0	17	20–30
"Deficient" + 0.01 M KH₂PO₄	156–167	14.8	82	75–85
"Deficient" + 0.05 M KH₂PO₄	76–89	1.5	8	20–30

* 1.8×10^7 per ml.
† Estimated from figure 1.
‡ Tryptone, 1 per cent; yeast extract, 0.3 per cent; glucose, 0.2 per cent.
§ Same as "complete" medium minus tryptone.

TABLE 2

The influence of calcium chloride on the adsorption of virus by S. lactis 122-4

MEDIUM	PLAQUE COUNT OF FREE VIRUS DUPLICATE PLATES	VIRUS ADSORBED PER ML × 10⁷	PERCENTAGE OF INITIAL VIRUS* ADSORBED	LYSIS OF CELLS
"Complete"	87–76	9.8	86	+
"Deficient"	43–60	1.1	10	−
"Deficient" + 0.001 M CaCl₂	32–53	2.9	25	−
"Deficient" + 0.01 M CaCl₂	149–163	8.3	73	+
"Deficient" + 0.02 M CaCl₂	76–93	9.7	85	+
"Deficient" + 0.1 M CaCl₂	46–58	1.0	9	−

* 11.4×10^7.

TABLE 3

The influence of sodium citrate on the adsorption of virus by S. lactis 122-4

MEDIUM	PLAQUE COUNTS OF FREE VIRUS DUPLICATE PLATES	VIRUS ADSORBED PER ML × 10⁶	PERCENTAGE OF INITIAL VIRUS* ADSORBED	LYSIS OF CELLS
"Complete"	138–113	62	71	+
"Deficient"	348–300	22	25	−
"Deficient" + 0.005 M sodium citrate	134–132	60	69	−
"Deficient" + 0.01 M sodium citrate	225–221	42	49	−
"Deficient" + 0.02 M sodium citrate	292–315	26	30	−
"Deficient" + 0.05 M sodium citrate	312–322	24	27	−

* 87×10^6 per ml.

virus ratio is low (6). If yeast extract (Difco) is added to the "deficient" medium to give a total of 1 per cent, virus production approaches that obtained in the "complete" medium. This is shown in experiment 5 of figure 2 and in table 4.
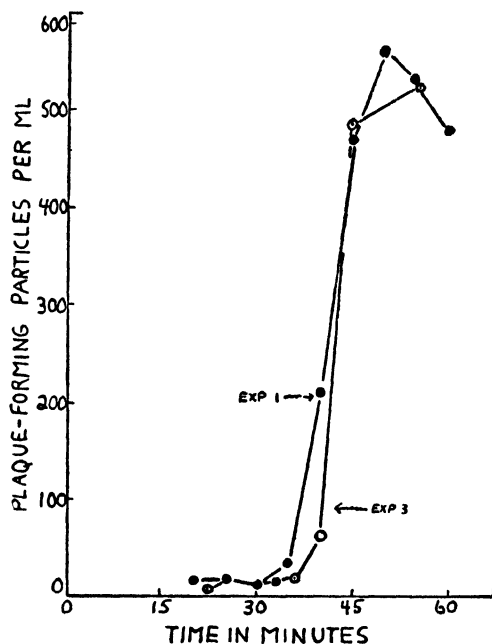
Figure 4. One-step growth curves of virus on *S. lactis* 122–4. Experiments 1 and 3 in "complete" medium at pH 7.0 and 30 C.
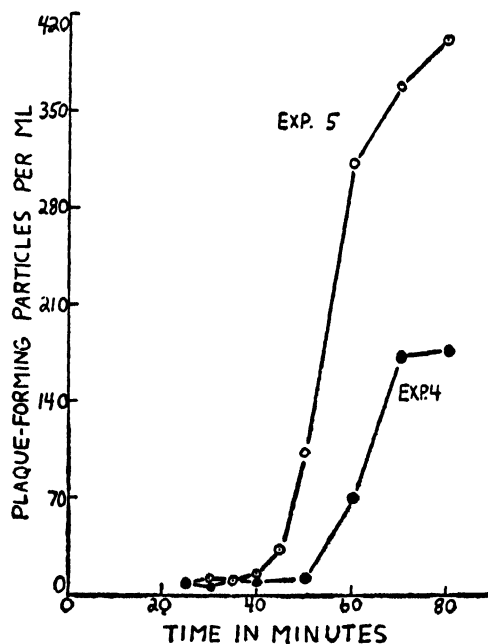
Figure 5. One-step growth curves of virus on *S. lactis* 122–4. Experiment 4 in "deficient" medium at pH 7.0 and 30 C. Experiment 5 in "deficient" medium plus 0.7 per cent yeast extract at pH 7.0 and 30 C.

However, the latent period of virus growth was about 10 minutes longer than it was in the tryptone medium. Thus it seems that on a weight basis yeast extract does not completely replace tryptone for virus production.

The effect of sodium citrate on virus production. As shown in figure 3, sodium citrate did not allow lysis of cells by virus. However, bacterial growth was inhibited to some extent even at the lower salt concentrations. Table 3 indicates that virus is readily adsorbed in the presence of 0.005 M sodium citrate. To determine whether virus was produced in the absence of bacterial lysis, one-step growth experiments were made in both "complete" and "deficient" media containing 0.005 M sodium citrate. In "deficient" medium no increase in virus was noted over a period of 93 minutes (table 4, expt. 6). After a latent period of 60 minutes, there was a small increase in virus in the "complete" medium (expt. 8). The final to initial virus ratio was about 4, and the average burst size was 5.

TABLE 4

Characteristics of the growth of virus on S. lactis 122-4

EX-PERI-MENT NO.	MEDIUM	MULTI-PLICITY OF INFEC-TION	MEAN LATENT PERIOD IN MINUTES	MEAN RISE PERIOD IN MINUTES	AVERAGE BURST SIZE PER INFECTED CELL	RATIO OF FINAL TO INITIAL VIRUS
1	"Complete".	3	32	17	66	18
3	"Complete"	3	31	15	77	21
4	"Deficient"	4	55	10	24	6
5	"Deficient" + 0.7% yeast extract.. ..	3	42	15	47	13
6	"Deficient" + 0.005 M sodium citrate..	1	—	—	<1	0.5
7	"Deficient" + 0.005 M sodium citrate + 0.02 M CaCl₂.	1	—	—	<1	0.5
8	"Complete" + 0.005 M sodium citrate..	1	60	10	5	3.5
9	"Complete" + 0.005 M sodium citrate + 0.02 M CaCl₂......	1	48	8	6	4.7

An attempt to relieve the inhibition of virus production by the addition of calcium chloride to give a 0.02 M solution was unsuccessful. Sodium citrate and calcium chloride were added to both "complete" and "deficient" media immediately before the experiments were begun. Calcium had no effect on increasing virus production in "deficient" medium (table 4, expt. 7), and the only observable effect in "complete" medium (table 4, expt. 9) was a reduction in the latent period of virus growth. Assays for virus were made up to 80 minutes. However, it was noted that after a long incubation period the cells in the tubes to which calcium chloride was added were completely lysed. The multiplicity of infection in the experiments conducted in media containing sodium citrate was about 1. Under these conditions many cells are not infected. Poisson's formula for calculating the fraction of uninfected bacteria is not applicable since the efficiency of plating is rather low (Cherry and Watson, 1949). However, even if only 50 per cent of the bacteria were infected the results would not be changed significantly. It appears that sodium citrate greatly depresses virus production

and that this effect can be reversed by the addition of calcium chloride only after a very long lag period, if at all. Virus assays throughout the course of the experiments gave no indication that citrate, at the levels used, had any appreciable effect in accelerating virus inactivation.

DISCUSSION

The average yield of virus obtained per infected cell of *S. lactis* 122-4 under the experimental conditions used was lower than the burst sizes that have been determined for the T series of viruses for *E. coli*. Delbrück (1946) reported burst sizes ranging from 120 to 300 for the seven viruses of the coli group. It is quite possible that other media may increase the yield of viruses for *S. lactis* above the burst size of 70 determined in tryptone yeast extract broth. The tendency of *S. lactis* cells to grow in short chains makes it difficult to determine absolute burst size values.

Tryptone contributed approximately a 3-fold stimulation to both adsorption and synthesis of virus for *S. lactis* 122-4. On a weight basis, yeast extract completely replaced tryptone for virus adsorption but could not be entirely substituted for tryptone for virus production. In view of the studies (Cohen, 1949) that have been reported on the growth requirements of the T series of viruses for *E. coli*, it is probable that this is an expression of similar requirements for the *S. lactis* host-virus system. Further studies along these lines are in progress. The stimulatory effects on virus adsorption of both 1 per cent yeast extract and 1 per cent tryptone are believed to be the result of the salts that they contain. The increased adsorption of virus occurring when salts are added to media deficient for virus adsorption is the evidence for this belief. The mechanism by which electrolytes produce these effects are obscure. The studies of Greenstein *et al.* (1947) on the importance of salts in the enzymatic degradation of nucleic acids by extracts of animal tissues suggest the possibility that adsorption of virus to the cell surface could be mediated by salt-activated enzymes. Anderson (1948) showed that the characteristics of activation of T_4 virus for adsorption on its host were not incompatible with an enzymatically controlled mechanism. It seems unlikely that the effect of electrolytes on adsorption of virus by *S. lactis* 122-4 can be entirely explained by purely physical phenomena involving the electrokinetic potential of cell and virus.

SUMMARY

The adsorption of virus by *Streptococcus lactis* 122-4 was about 3 times as great in a medium consisting of 1 per cent tryptone, 0.3 per cent yeast extract, and 0.2 per cent glucose as in the same medium when tryptone was omitted.

On a weight basis, yeast extract replaced tryptone for virus adsorption.

Salt stimulation of cellular lysis by virus can be used as a method of screening electrolytes for effects on virus adsorption. Electrolytes were most stimulating for both cellular lysis and virus adsorption at concentrations ranging from 0.005 M to 0.05 M. Potassium phosphate, potassium, sodium and calcium chloride, magnesium sulfate, and sodium acetate promoted lysis of the host cells

according to their efficiency in promoting virus adsorption, as measured by plaque count assays.

Sodium citrate did not permit lysis of host cells by virus although it allowed maximum adsorption and did not inactivate the virus. Virus synthesis was greatly reduced in the presence of 0.005 M sodium citrate. This level of salt is somewhat inhibitory to multiplication of the bacteria. Addition of 0.02 M calcium chloride did not release the citrate inhibition except to permit cellular lysis after a very long lag period.

The average yield of virus per infected cell of *S. lactis* 122-4 in tryptone yeast extract medium at pH 7.0 and 30 C was about 70 plaque-forming particles. The burst size was reduced by a factor of three when tryptone was not present in the medium even though adsorption of virus was maintained at a high level by the addition of potassium phosphate to give a 0.01 M concentration. On a weight basis, yeast extract only partly replaced tryptone for virus synthesis. This suggests that this host-virus system requires growth factors that are not entirely supplied by yeast extract.

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INHIBITION OF YEAST HEXOKINASE BY HOMOLOGOUS ANTISERUM[1,2]

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As a continuation of our studies on the effects of immune sera on cellular metabolism (Sevag and Miller, 1948), we have investigated the effect of homologous rabbit immune serum on the activity of hexokinase isolated from yeast. This report represents the results of this study.

It is known (Von Euler and Adler, 1935; Meyerhof, 1935; Colowick and Kalckar, 1943) that hexokinase catalyzes the transfer of one phosphate group from adenosinetriphosphate to glucose with the formation of glucose-6-phosphate. In this reaction an alcohol hydroxylic hydrogen is converted to a more acidic hydrogen. As will be seen, immune serum added to the complete reaction system at the beginning of the experiment or after the reaction has started, completely inhibits the activity of hexokinase.

EXPERIMENTAL METHODS

Hexokinase was isolated from Fleischmann's bakers' yeast according to the method of Kunitz and McDonald (1946), and the dialyzed "0.72" fraction was used for immunization and for activity tests with various sera. Uniform enzyme activity was maintained by preserving the preparation in 1 per cent glucose solution in the frozen state. This preparation compared with the five-times-crystallized hexokinase (specific activity, 1,440) of Kunitz and McDonald is only 10 per cent pure.

Immunization. Method 1: Four large male rabbits were immunized in the following manner: Five subcutaneous injections, given at 3- and 4-day intervals, varied from 1 to 3.5 ml. The protein content per ml was about 3 mg, which corresponds to 0.3 mg of 5-fold crystalline hexokinase. Sera collected 10 days after the last injection completely inhibited hexokinase activity.[3] To maintain the antibody content of these rabbits at a satisfactory level, a second series of four injections was given 2½ months later. The rabbits were exsanguinated, and the sera were pooled, inactivated (56 C for 30 minutes), and kept in the frozen state without a preservative.

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[1] This is the second article in a series of studies on the effect of immune reactions on the metabolism of bacteria.

[2] This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

[3] In our experience, dialyzed crystalline hexokinase was not suitable for a long course of immunization because of its instability. The activity of such a purified preparation was completely inhibited by antiserum prepared against 10 per cent pure hexokinase.

Precipitation and complement-fixation tests performed on all immune sera showed low titers. However, these sera, in contrast to the immune serum described below, strongly inhibited hexokinase activity.

Method 2: A rabbit was immunized by the intramuscular injection of a 12-ml emulsion of hexokinase (8 mg protein content) in Falba mineral oil using a modification of Freund and his co-workers' procedure (Freund and Bonanto, 1944; Halbert, Smolens, and Mudd, 1945). The emulsion was prepared by being stirred in an electric mixer in the cold for about 3 minutes. Two volumes of 6 ml each of the emulsion were injected at different sites at the same time. A month later the serum from this rabbit was tested. It showed precipitating and
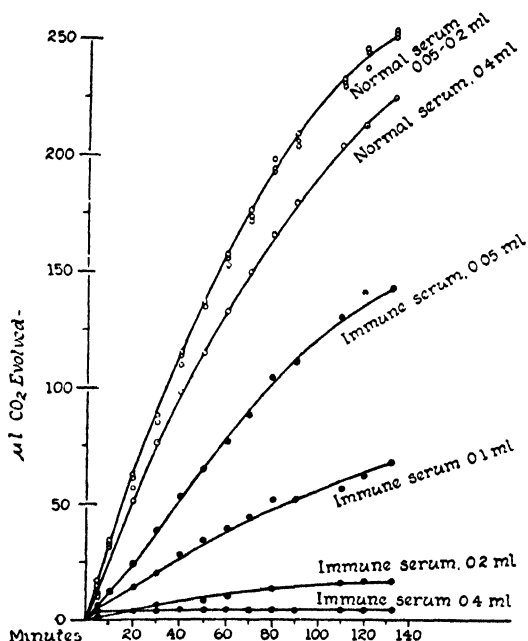
Figure 1. Inhibitory effect on yeast hexokinase by homologous rabbit antiserum added before the reaction had started.

complement-fixing titers higher than the sera described above but had no hexokinase-inhibiting activity. Under these conditions, hexokinase was either inactivated, producing antibodies incapable of inhibiting the active enzyme, or the amount injected was insufficient to produce enzyme-specific antibody.

Determination of the inhibitory effect of immune sera on hexokinase activity. The methods used were (a) the measurement of the carbon dioxide evolved and (b) a direct titration of the hydrogen ions produced in the reaction. The results by both methods were analogous. The data reported here were obtained by the manometric measurement of carbon dioxide evolved. The method used was that of Colowick and Kalckar (1943) and Berger et al. (1946) adapted to our conditions.

In our experiments (figure 1) the main compartment of the Warburg flask contained 0.27 ml of 0.144 M sodium bicarbonate, 0.14 ml of 0.50 M glucose, 0.10 ml of hexokinase (1 mg protein per ml), 0.05 to 0.4 ml of serum, and 0.77 to 1.12 ml of 0.9 per cent sodium chloride, pH 7.5. The side arm contained 0.58 ml of 0.04 M ATP (sodium salt, pH 7.5), 0.07 ml of 0.2 M magnesium chloride, and 0.07 ml of 0.1 M sodium bicarbonate. The final volume of the system was 2.4 ml.
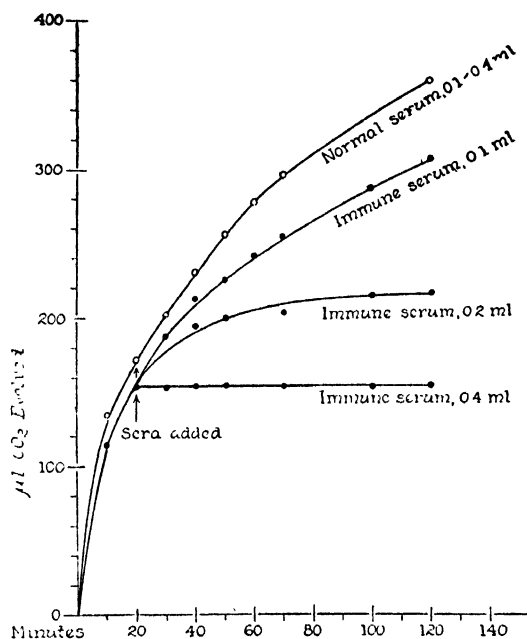
Figure 2. Inhibitory effect on yeast hexokinase by homologous rabbit antiserum added after the reaction had proceeded for 20 minutes.

The experiment, the results of which are presented in figure 2, was conducted in the following manner: Double-armed flasks were used. Side arm (1) contained the serum and saline, total volume, 0.7 ml. Side arm (2) contained 0.58 ml of ATP. The main compartment of the flask contained 0.07 ml of magnesium chloride, 0.32 ml of sodium bicarbonate, 0.14 ml of glucose, 0.1 ml of hexokinase, and 0.49 ml of saline; pH 7.5. The ATP was added from side arm (2) after equilibration of the temperature was reached. The contents of side arm (1), immune or normal serum, were added after the reaction had been in progress for 20 minutes. The flask used as a thermobarometer contained all reagents except hexokinase and serum.

EXPERIMENTAL RESULTS

The results presented here show that rabbit immune serum prepared against yeast hexokinase completely inhibits the latter's activity. The high specificity of this reaction is indicated by the fact that (a) a rabbit immune serum prepared against the same enzyme preparation suspended in Falba mineral oil, even though it produced an immune serum with higher precipitating and complement-fixing titer, was incapable of exercising an inhibitory effect on the hexokinase

activity; (b) a rabbit immune serum prepared against streptococcal hyaluroni-
dase[4] likewise failed to inhibit hexokinase activity; and (c) an aqueous extract
of rat brain (8 ml of chilled distilled water per rat brain) as the source of mam-
malian hexokinase, when substituted for yeast hexokinase, was not inhibited by
rabbit normal and antiyeast hexokinase sera. Three-tenths ml of brain extract
was approximately equivalent in activity to 0.1 ml of our yeast hexokinase
preparation.

Four-tenths ml of immune serum completely inhibits the activity of 0.1 ml
of hexokinase solution. The inhibitions by 0.05 ml, 0.1 ml, and 0.2 ml of immune
serum are, respectively, 42, 73, and 94 per cent (figure 1). The data presented in
figure 2 show a similar qualitative relationship with respect to the inhibition
with various volumes of immune serum. The optimal volume (0.4 ml) of im-
mune serum, added at a time when hexokinase activity was at its peak, brought
about an immediate inhibitory effect, which persisted through the 2-hour period.
This relationship between an enzyme and its homologous antibody would ap-
pear to be analogous to the inhibition of enzymes with specific inhibitors. A spe-
cific antiserum inhibitory for yeast D-glyceraldehyde-3-phosphate dehydrogenase
has been described by Krebs and Najjar (1948). For a discussion of immune
reactions involving other enzymes see Sevag (in press).

SUMMARY

Antiserum against yeast hexokinase has been produced in the rabbit. This
serum, added to the reaction system at the beginning or after the reaction has
progressed for 20 minutes, inhibits the activity of the enzyme. Under optimal
conditions the inhibitory effect of the antiserum is immediate.

Yeast hexokinase antiserum had no effect on the activity of rat brain hexoki-
nase, nor did a rabbit antihyaluronidase serum show an inhibitory effect on yeast
hexokinase. These facts show a high degree of specificity of the inhibitory
action of the antiserum obtained.

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[4] Courtesy of Dr. T. N. Harris of the Children's Hospital, Philadelphia, Pennsylvania.

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LYSOGENESIS OF BACILLUS MEGATHERIUM[1]

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It has been questioned for a long time whether a virus-infected cell is capable of multiplying with that virus present internally; or, in other words, whether the infected cell is capable of carrying on its own metabolic functions simultaneously with those required for virus synthesis. Such a state is called lysogenesis and has been doubted by many workers. For instance, Demerec and Fano (1945) and Delbrück (1946) thought that lysogenesis in bacteria-bacterial-virus systems is more apparent than real. In reality, they thought that a lysogenic culture was one in which most cells were virus-resistant mutants. However, a few mutants were constantly changing back to virus-susceptible cells. Any phage carried along externally would infect these cells and multiply.

We have found that cells of *Bacillus megatherium* can multiply with virus present within them, and that such infected cells are resistant to lysis by external virus. We were able also to show that, by adding glucose to a medium relatively rich in nitrogen, we could rid the infected cell of its internal virus and thus return it to its susceptible state.

MATERIALS AND METHODS

In this work *B. megatherium* strain 1 (University of Toronto, Canada) and megatherium phage φ 16 B (University of Toronto, Canada) were used. Vegetative, lysogenic cultures were developed from spores experimentally infected by essentially the methods of Den Dooren de Jong (1931) and Cowles (1931). Infected spores were heat-shocked at 85 C and germinated in tryptone beef extract broth. The germinate was plated on nutrient agar, and a mottled colony, assumed to be lysogenic by the criteria of De Jong (1931), was isolated and cultured on an agar medium of tryptone and beef extract. Such a culture has been carried along in stock and used for subsequent experiments.

All media used in these experiments contained 3 g Difco beef extract and 5 g Difco tryptone in 1,000 ml distilled water. Glucose and agar were added in the required amounts when needed.

To show that our lysogenic strain produced virus, equal amounts (0.1 or 0.2 ml) of a serially diluted suspension of a 24-hour lysogenic culture were put on the surface of each nutrient agar plate and spread by rotating the plate. These plates were incubated at 30 C. Virus titrations were made of the inoculum and

of the growth on the nutrient agar plates by the method of Hershey (1943). To assay the plates for external virus, the growth of each was suspended in 5 ml sterile broth; the cells were then removed by centrifugation, and the supernatant was titered.

Since some virus-infected cells have been shown to stain more basophilically than uninfected cells, we wished to show cytochemically that the lysogenic cells contained internal virus by adapting for this purpose the nuclear staining technique of Robinow (1942) as modified by Beumer and Quersin (1947). A lysogenic culture on plain tryptone was suspended in distilled water and autoclaved for 20 minutes at 15 pounds pressure. One-half ml of the autoclaved culture was treated with one-half of a solution of crystalline ribonuclease (Armour) in a water bath at 45 C for 1 hour to remove the gram-positive layer from the cell surface (Bartholomew and Umbreit, 1944). The enzyme solution contained 0.1 mg enzyme per ml. Three loopfuls of this digest were put on a slide, air-dried, and fixed in the vapors of 2 per cent osmic acid for 2 minutes. After thorough washing under tap water, the preparation was treated in 4 N HCl at 56 C for 20 minutes and washed under running tap water for at least 5 minutes. Then the preparation was stained for 1 hour with Giemsa solution (Eimer and Amend) and diluted to 2.5 per cent with distilled water. The slides were subsequently washed under tap water, dried, and examined under oil immersion. In all experiments a control of an uninfected culture of B. megatherium was run simultaneously with the lysogenic culture. This technique has been shown to be specific for desoxyribonucleic acid (DRNA) by Vendrely and Lipardy (1946) and Tulasne and Vendrely (1947).

Cohen (1948) has shown that bacterial cells infected with virus are richer in DRNA than are uninfected cells. Consequently we attempted to show chemically that virus was present internally in the lysogenic cells by performing a DRNA analysis. A lysogenic culture and an uninfected culture on nutrient agar were harvested separately in distilled water at about 14 hours. The suspensions were autoclaved for 20 minutes at 15 pounds pressure to inactivate all enzymes and coagulate the proteins. The cultures were then washed three times in distilled water and lyophilized. After the dry weight of the lyophilized cultures had been determined, they were suspended in 5 per cent trichloroacetic acid to make a 0.5 per cent suspension. To extract the DRNA, the suspensions were then heated to 90 C for 15 minutes, allowing 1 minute for coming up to temperature. Dilutions of standard DRNA (Nutritional Biochemicals Company) in 5 per cent trichloroacetic acid were simultaneously heated to 90 C for 15 minutes. The suspensions were then spun down and known volumes of supernatant analyzed for DRNA by the Dische method (1930). The percentage of DRNA in the dry weight of the bacterial cells was calculated from the formula:

$$\frac{\text{mg per ml of DRNA in supernatant}}{\text{5 mg per ml bacterial cells in suspension}} \times 100$$

The DRNA content in the standard DRNA solution was determined from micro-Kjeldahl nitrogen. According to the nitrogen value given by Cohen (1948),

16.9 mg of nitrogen are contained in 100 mg DRNA, the DRNA concentration in the standard solution being calculated from this value.

To show the effect of glucose on the lysogenic strain, cultures were inoculated onto nutrient agar containing 1 per cent glucose and analyzed after 14 hours by the cytochemical and chemical techniques just described. Plain nutrient agar slants inoculated with the lysogenic strain were analyzed simultaneously with the others.

<div align="center">RESULTS</div>

The lysogenic culture, which we isolated, produced virus during its growth, as shown in table 1. No increase in external virus occurred after the second day.

The cytochemical test showed that the lysogenic culture contained DRNA distributed throughout the cell, but the uninfected cell contained DRNA only in a more central region. The lysogenic cultures stained an even purple, whereas the uninfected cells showed a purple-staining, oval, central body surrounded by

<div align="center">TABLE 1</div>
<div align="center">*Virus production of lysogenic culture on solid medium*</div>

DAY OF OBSERVATION	VIRUS TITER PER ML OF SUSPENSION AFTER WASHING OFF CULTURE*
Initial	4×10^5
1	2.4×10^5
2	2.1×10^7
3	6.8×10^6
4	5.1×10^6
5	8.3×10^6

* In a constant volume (5 ml) of suspension.

pink-staining material. Electron micrographs of 14-hour-old autoclaved cells showed that the pink-staining area was not a capsule but an integral part of the cell structure, since this material contained distinct cross walls. Lysogenic cells were not distinguishable from uninfected cells in the electron micrographs.

Chemically it was possible to show that the lysogenic cells contained about two times as much DRNA as the uninfected cells (table 2). That a higher than normal DRNA content is indicative of virus synthesis in the bacterial cell can be inferred from the work of Cohen (1948).

If the infected cells were exposed to the usual nutrient agar medium that contained 1 per cent anhydrous glucose (Mallinckrodt), staining after 14 hours of exposure showed a complete loss of internal virus. The cells showed the same cytological picture as the uninfected cells. Suitable controls were run in these experiments.

Chemical analyses of lysogenic cultures exposed to 1 per cent glucose for 14 hours gave similar results. A loss of DRNA to about one-half the initial value was obtained. The results are listed in table 2.

We were able to show that glucose does not act selectively for mutants of

susceptible cells in an otherwise resistant culture. By plating a lysogenic culture by the spread technique on ordinary nutrient medium and nutrient medium containing 1 per cent glucose, the same number of colonies was obtained in both cases (table 3). A far smaller number of colonies should have been obtained on the glucose medium if it had selective action.

Glucose does not inhibit lysis of uninfected cells by its virus. If such cells, previously exposed to 1 per cent glucose in two successive transfers, are exposed

TABLE 2

Percentage of DRNA in dry uninfected cells, lysogenic cells, and recovered cells of B. megatherium

EXPERIMENT	PERCENTAGE OF DRNA IN DRIED CELLS		
	Uninfected cells	Infected cells	Recovered cells
A	1.5	3.0	—
B	1.1	2.3	—
C*	—	2.9	1.4
D†	—	2.4	1.7

* Cells 15 hours old at time of harvest.
† Cells 17 hours old at time of harvest.

TABLE 3

Test for the selective effect of glucose on the lysogenic culture

EXPERIMENT	DILUTION	NO. OF COLONIES ON 1.0 PER CENT GLUCOSE AGAR	NO. OF COLONIES ON PLAIN NUTRIENT AGAR
A	0.2×10^{-3}	110	130
B	0.1×10^{-3}	536	258

TABLE 4

Effect of glucose on lysis of a normal culture of B. megatherium by phage

EXPERIMENT	VIRUS TITER ON PLAIN NUTRIENT MEDIUM	VIRUS TITER ON NUTRIENT MEDIUM WITH 1.0 PER CENT GLUCOSE*
A	1.7×10^7	1.3×10^7
B	1.3×10^7	1.3×10^7

* Susceptible cells previously exposed to 1.0 per cent glucose; glucose added to all media used in the titration.

to virus in the presence of glucose and titered by the Hershey method, approximately the same number of plaques are obtained as without glucose (table 4).

A lysogenic culture of *B. megatherium* can be distinguished from an uninfected culture on nutrient medium by its ground-glass appearance and iridescence in transmitted light. The uninfected culture is opaque under these conditions. The lysogenic cells are arranged in parallel in a colony, but the uninfected cells are arranged in criss-cross fashion. Glucose in the medium changes the ground-glass

appearance of the lysogenic culture into opaque growth. If a lysogenic culture is exposed to glucose, the resultant culture when mixed with stock virus will again lyse.

DISCUSSION

These experiments indicate that cells of *B. megatherium* can actually multiply with phage present within them and that such infected cells are resistant. This observation makes it possible to interpret differently the findings of other workers, who explained lysogenesis on a genetic basis.

It was possible to demonstrate that lysogenic cells when grown on a nutrient medium containing glucose would lose their internal virus. These conclusions were drawn from analyses of the DRNA content of uninfected and infected as well as lysogenic cells exposed to glucose. The DRNA content of lysogenic cells before exposure to glucose was decidedly higher than that of uninfected cells or that of lysogenic cells after exposure to glucose. The return of susceptibility was concurrent with a disappearance of external virus. In other words, no more virus was synthesized by the susceptible cells.

The effect of glucose may be a metabolic one since there was a loss of internal virus within 15 hours. If the pH of the medium during growth is the key factor, the lysogenic cells must be very sensitive to slight pH changes on the acid side. The pH of the glucose medium falls to about 6.2 in 1 to 2 days. It should also be mentioned that a glucose concentration of 0.1 per cent does not eliminate the lysogenic state. Further work is being done in an attempt to elucidate this problem.

The resistance of lysogenic cells to lysis by external phage can be explained by the interference phenomenon, discovered for bacteriophages by Delbrück and Luria (1942).

SUMMARY

Evidence for true lysogenesis in *Bacillus megatherium*, strain 1, with megatherium phage φ 16 B has been accumulated. Cytochemical and chemical data were obtained to clarify this point.

Glucose, added to the normal medium, was shown to render virus-resistant, lysogenic cells susceptible to the virus with which they were originally infected. Such cells showed a decrease in desoxyribonucleic acid after exposure to glucose. Glucose does not prevent lysis of susceptible cells exposed to virus.

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A STUDY OF THE LACTIC ACID BACTERIA THAT CAUSE SURFACE DISCOLORATIONS OF SAUSAGES

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Sausages prepared from cured, comminuted meats (wieners, bolognas, etc.) are subject to several types of spoilage, the majority of which are manifested by a visual change in color of the product. Most, but not all, of these discoloration problems are known to be of microbial origin. One of the types of spoilage is due to bacterial contamination on the surface of the sausage after processing, which, under appropriate conditions, results in a change in color of the cured meat pigment to a dull green, and thence to a faded color. This change may occur on the uncut surface, as in the case of wieners or other small sausages, or on the sliced surface of such products as bolognas, luncheon meat loaves, and other cold cuts.

The first evidence of spoilage is the appearance of small greenish spots on the damp surfaces of the product, which tend to spread and cover the entire surface in due time if favorable conditions are present. Accompanying the surface discoloration, a slight "greasy" or slimy appearance of the product may be noted in most cases. The discoloration usually penetrates the sausage to a depth of 1 to 2 millimeters. A freshly cut surface invariably reveals an interior that is normal in appearance, odor, and flavor.

Bacteriological evidence indicates that this type of discoloration on the various types of cured meat sausages results from surface contamination after the sausages have been smoked and cooked. Very large numbers of bacteria are found on those areas showing discoloration, but very few viable bacteria may be found in samples obtained from the interior.

It has been reported that the bacteria found to be associated with this type of sausage discoloration are either heterofermentative lactobacilli or members of the genus *Leuconostoc* (Niven, Castellani, and Allanson, 1948). Further studies indicate that these microorganisms comprise rather homogeneous varieties or species within their respective genera and are distinct from any species hitherto described.

CULTURES

The cultures used in this study were obtained from natural outbreaks of surface discoloration on sausages. Scrapings from the discolored areas were plated onto suitable agar media and incubated for 3 days at 30 C. The most satisfactory plating media found were trypticase soy agar (B.B.L.) or blood agar. Representative colonies were picked into a glucose, beef infusion broth and streaked back onto cut surfaces of freshly steamed, normal-appearing wieners.

These inoculated wieners were then incubated in moist chambers at 30 C. Those cultures that discolored the steamed wieners within 24 hours were considered to be associated with the natural discoloration of the sample examined.

A large variety of sausages prepared from cured meats showing surface discoloration have been examined for the presence of "green-producing" bacteria. In every case in which the discoloration was of microbiological origin, the green-producing bacteria were present in very large numbers, usually in great excess over any other type of microorganism found. Thirty of these greening bacteria were selected at random from ten discolored samples for extended study. These samples originated from six widely separated geographical locations in the eastern half of the United States. Twenty of these 30 cultures were found to be members of the genus *Lactobacillus* (subgenus, *Betabacterium*). The remaining 10 cultures were members of the genus *Leuconostoc* (*Betacoccus*).

CHARACTERISTICS OF THE LACTOBACILLUS STRAINS

Morphologically the *Lactobacillus* cultures are short, straight rods (2 to 4 microns in length) with rounded ends, occurring either singly or in pairs. They are strongly gram-positive, as viewed in films prepared directly from the original samples as well as from pure cultures. Little granulation or marked variation in morphology was noted under any of the cultural conditions tested.

Growth on all plating media tested was rather slow. Colonies were either barely perceptible or not yet visible after 24 hours at 30 C. After 3 days' incubation on a glucose, beef infusion agar the colonies were smooth, compact, and approximately 0.5 millimeter in diameter. Growth on blood agar was usually inferior, but was more easily detected because of the green zone surrounding the individual colonies.

In glucose broth media, uniform turbidity, with some sediment, developed after 48 hours at 30 C. Growth was considered to be rather slow, with maximum turbidity usually inferior to that produced by some of the better known heterofermentative lactobacilli tested under the same conditions.

All cultures adhered to the characteristics recognized for the genus *Lactobacillus* in that they were nonmotile, nonpigmented, and catalase-negative and failed to reduce nitrates. Incubation under anaerobic conditions and under 10 per cent CO_2 appeared to improve growth to a slight degree.

Table 1 presents the physiological characteristics determined for the 20 cultures. Perhaps because of their slow rate of growth, a great deal of difficulty was encountered with the usual cultural methods for determining gas production by these microorganisms. However, dependable results were obtained with the use of Eldredge tubes. A basal medium consisting of 1 per cent (Difco) tryptone, 0.5 per cent (Difco) yeast extract, 0.2 per cent dipotassium phosphate, and 0.5 volume beef infusion was employed, in which 1 per cent glucose was added aseptically prior to inoculation. In this medium at 30 C, the cultures fermented 0.7 to 0.9 per cent glucose within 10 days. Calculated on a weight basis, all cultures produced 19 to 24 per cent carbon dioxide from the sugar fermented.

In a separate experiment three of the greening lactobacillus strains produced a lactic acid yield of only 36 to 40 per cent, based upon the glucose fermented.

Lactic acid was determined quantitatively by the method of Barker and Summerson (1941). Zinc salts of the lactic acid produced from glucose by these three strains were isolated for the purpose of determining the optical form produced. In each case the inactive form of lactic acid was produced. These determinations tend to confirm the heterofermentative nature of the lactobacillus strains. No

TABLE 1

Physiological characteristics of the sausage-greening microorganisms

	LACTOBACILLUS GROUP (20 STRAINS)	LEUCONOSTOC GROUP (10 STRAINS)
CO_2 from glucose.....	+	+
Polysaccharide from sucrose	14+; 6−	−
Greening on blood agar.... . ..	+	+
Final pH, glucose broth.......... . .	4.6–4.8	4.6–4.9
Litmus milk.	No change	Sl. acid
Lactic acid from glucose	DL	D(−)
Growth:		
5 C.... .	+	+
40 C.... . . .	±	±
6.5% sodium chloride . ..	+	+
10.0% sodium chloride	−	+
Hydrolysis:		
Sodium hippurate...... .. .	−	−
Esculin . .	−	+
Arginine	−	−
Gelatin .	−	−
Starch..	−	−
Fermentation:		
Xylose.....	−	−
Arabinose	−	+
Mannose..	+	+
Fructose	+	+
Galactose	−	+
Lactose........	−	+
Maltose.......	+	+
Sucrose....	15+; 5−	+
Trehalose........	4+; 16−	+
Raffinose..	−	+
Inulin........	−	−
Glycerol.	−	−
Mannitol..........	−	−
Sorbitol.............	−	−

attempts were made to identity other fermentation products of these microorganisms.

When streaked upon a 5 per cent sucrose gelatin agar medium, 14 of the 20 cultures produced large mucoid colonies very similar to those produced by certain *Leuconostoc* cultures. Of the 6 strains that failed to possess this characteristic, 5 were unable to ferment sucrose.

The optimum temperature for growth appeared to be approximately 30 to

32 C. All cultures grew at 37 C but none were able to grow at 45 C. The maximum temperature for growth appeared to be 40 C, as evidenced by the fact that the cultures grew poorly, or not at all, at this temperature. The lower temperature limit for growth was below 5 C. All cultures developed visible turbidity in broth culture at 3.5 C within 1 week.

The greening lactobacilli were relatively tolerant to salt. All strains grew in a glucose medium containing 6.5 per cent sodium chloride. Growth occurred in the presence of 8.0 per cent sodium chloride, but was markedly reduced. Higher concentrations of salt completely inhibited growth.

The fermentation pattern of these cultures was rather striking. Only glucose, fructose, mannose, and maltose were fermented by all of the strains. The majority fermented sucrose, whereas only a few fermented trehalose. None of the cultures were able to ferment the pentoses, galactose, lactose, or any of the higher alcohols tested.

CHARACTERISTICS OF THE LEUCONOSTOC STRAINS

The individual cells of the ten *Leuconostoc* cultures were either spherical (approximately 1 micron in diameter) or ovoid in shape, occurring in short to moderately long chains when viewed from broth cultures. Some chains contained as many as 20 cells. In films prepared directly from discolored sausage samples, these microorganisms appear in much shorter chain lengths. The majority may be present as individual cells or in pairs; consequently, the presence of large numbers of *Leuconostoc* microorganisms on the original product may be easily overlooked. All cultures were strongly gram-positive.

Growth on glucose agar or blood agar plating media was much more rapid for these than for the *Lactobacillus* cultures. Within 2 days at 30 C the individual colonies were smooth, compact, and approximately 1 millimeter in diameter. Blood agar colonies were surrounded by strong green zones.

Within 24 hours at 30 C, glucose broth cultures showed moderately heavy turbidity. After 48 hours the cells tended to settle out. All cultures were nonmotile, nonpigmented, and catalase-negative and failed to reduce nitrates.

The physiological characteristics of these cultures are also presented in table 1. With the same techniques described for the *Lactobacillus* cultures, the 10 *Leuconostoc* strains were found to produce 15 to 24 per cent carbon dioxide from the sugar fermented. Lactic acid yields from glucose fermentation by two of the strains were approximately 25 and 35 per cent. The lactic acid produced by these two strains was *levo*-rotatory, which is in line with the better known species within this genus.

Somewhat striking was the fact that none of the 10 greening *Leuconostoc* cultures produced mucoid colonies on sucrose agar. Repeated attempts to demonstrate this phenomenon under different conditions failed. No polysaccharide synthesis from sucrose occurred under anaerobic conditions, or in sucrose gelatin stab cultures as described by Hucker and Pederson (1930); yet, all cultures fermented sucrose.

The greening *Leuconostoc* cultures showed practically the same temperature

limits for growth as the *Lactobacillus* cultures described. They grew slowly at 3.5 C; poor or no growth occurred at 40 C. In comparison with the lactobacilli, the *Leuconostoc* cultures were tolerant to high salt concentrations. All strains grew in the presence of 10 per cent sodium chloride; some grew moderately well in concentrations as high as 12 per cent.

All of the ten strains fermented arabinose, the hexose sugars, the disaccharides, and raffinose. Xylose and the higher alcohols were not fermented.

SEROLOGICAL STUDIES

One of the *Lactobacillus* strains was inoculated into 100 ml of beef infusion broth containing 0.1 per cent glucose and incubated for approximately 30 hours at 30 C. The cells were sedimented, resuspended in 5 ml saline, and heat-killed at 56 C for 1 hour. The volume was then adjusted to 25 ml with saline. One ml of this prepared antigen was inoculated intravenously into rabbits daily for 5 days each week. Ten days after the fifth series of injections the rabbits were bled and the serum was collected in the usual manner.

Extracts of 18 of the greening lactobacilli were prepared using minor modifications of the original method of Lancefield (1933). Of the 18 extracts tested, all gave a strong precipitin test with the serum, thus demonstrating the serological homogeneity of the cultures. No cross reactions were obtained with any other cultures tested. A serum prepared by use of a second greening *Lactobacillus* culture as an antigen gave identical results.

A serum was also prepared from one of the *Leuconostoc* cultures. Of the 10 *Leuconostoc* extracts tested, five gave strong precipitin tests equal in intensity to that of the homologous strain. The remaining five extracts also gave positive precipitin reactions with the serum, though much weaker than that obtained with the homologous strain. Serum obtained by an additional four series of injections failed to produce any increase in intensity of reaction with these five extracts.

Reciprocal precipitin tests between the *Lactobacillus* and *Leuconostoc* groups failed to reveal any cross reactions.

DISCUSSION

The number of cultures representing the two groups of microorganisms described is admittedly small. However, considering the widely separated geographical sources from which the cultures were obtained, it would seem more than coincidental that they comprise such remarkably homogeneous groups within their respective genera. The majority of the cultures included in this study were isolated within a period of approximately nine months. However, the chances of their having originated from a common source would be exceedingly small.

Taxonomically, the lactobacilli would fit into the heterofermentative group characterized by their low temperature limits of growth. They are perhaps more closely related to *Lactobacillus brevis* than any other described species. However, and this is substantiated by a comparative study of several heterofermentative lactobacilli received from Dr Carl S. Pederson, these cultures are easily dis-

tinguished from any of the known species. Of the few tested, none of the known heterofermentative *Lactobacillus* strains were capable of discoloring sausages in pure culture.

Of particular interest was the ability of the sucrose-fermenting *Lactobacillus* strains to produce mucoid colonies on sucrose agar. Only one sucrose-fermenting strain failed to synthesize a polysaccharide from this disaccharide. This strain fermented sucrose slowly. In a study of the group as a whole, however, the percentage of the cultures that form mucoid colonies might be considered too low to be of great taxonomic value.

The synthesis of a polysaccharide from sucrose by lactobacilli has not been recognized to any great extent, although it may be a more common occurrence than is suspected. Dr. Pederson has called the authors' attention to the description of *Bacterium vermiforme* Ward, described in *Bergey's Manual of Determinative Bacteriology* (1948). This organism, later called *Betabacterium vermiforme* Mayer, appears to have been a slime-forming *Lactobacillus* that was isolated from the ginger beer plant fermentation. More recently, Kobayasha (1944) has described a microorganism, *Lactobacillus musicus*, isolated from tobacco leaves. This microorganism is reported to ferment sucrose into a viscous substance. The relationship of the sausage-greening lactobacilli to these slime-producing bacteria is unknown.

As mentioned previously, the greening lactobacilli grow rather slowly and have a comparatively high limiting pH in glucose culture media. On the other hand, these microorganisms appear to grow rapidly when inoculated onto sausages. This apparent discrepancy might be due to the existence of a nutritional deficiency in the artificial media used. Many different types of media and supplements have been tested in attempts to enhance the growth of these bacteria. These attempts have failed to divulge any significant information concerning the nutrition of these microorganisms. Furthermore, preliminary trials indicate that representative cultures of this group are capable of growing in a medium composed of chemically defined constituents. Therefore, it is unlikely that the cultures require an unrecognized growth factor. At the moment, no explanation can be offered for these anomalous observations.

The *Leuconostoc* cultures are taxonomically peculiar in that they ferment sucrose and yet do not form mucoid colonies on agar media containing this substrate. They also appear to be much more tolerant to sodium chloride than the described species of this genus. With the exception of the above-mentioned characters, these microorganisms might be considered to be related to *Leuconostoc mesenteroides*.

The serological studies might indicate the existence of a serological group, as well as type, relationship among the *Leuconostoc* cultures similar to that among the hemolytic and some other streptococci. However, the evidence presented is too meager to warrant serious consideration of this possibility. Also, conforming to the existing terminology for the streptococci, the 20 greening lactobacilli would appear to be members of a single type, rather than of a serological group.

The question arises as to the mechanism by which the sausages are discolored when these bacteria are allowed to grow extensively on the surface. The cured meat pigments (nitric-oxide myohemoglobin and nitric-oxide hemochromogen) can be easily oxidized, with accompanying change in color (Urbain and Jensen, 1940). Evidence has appeared (Jensen, 1944, 1945; Jensen and Urbain, 1936) which indicates that certain types of discoloration of sausages result from the microbial production of peroxides that react chemically with the cured meat pigment. Experiments of a preliminary nature indicate that the bacteria described here also discolor the sausages as the result of hydrogen peroxide production. Being members of the lactic acid group of bacteria, they are devoid of catalase. The catalase originally present in the sausage meats is inactivated by the curing ingredients and the smoking and cooking temperatures. Therefore, when these bacteria are the predominant microorganisms present on the sausage, little or no catalase may be present.

Under the conditions tested, cell suspensions of one of the greening lactobacilli were able to oxidize unknown substances in beef infusion with the production of hydrogen peroxide in concentrations up to 0.02 per cent. Experiments were conducted in which different concentrations of hydrogen peroxide were applied to freshly cut surfaces of sausages. When a liberal application of 0.03 per cent hydrogen peroxide was made, discoloration occurred after several hours similar to that produced microbiologically. Higher concentrations resulted in prompt and complete fading.

Few attempts were made to determine the substance, or substances, in beef infusion that were oxidized by the test culture. However, incubation with glucose resulted in the accumulation of low concentrations of hydrogen peroxide. In contrast to the "minute" and certain viridans streptococci that produce large quantities of hydrogen peroxide from butyrate (Niven, Evans, and White, 1945), the test *Lactobacillus* culture failed to oxidize this substrate.

If sausages are inoculated with the greening cultures and incubated anaerobically, no discoloration occurs, although abundant growth takes place. However, upon removal from the desiccator, the sausages discolor within a few hours.

Indirect evidence that the discoloration occurs as the result of hydrogen peroxide production was gained by the observation of a ham slice that had developed several green spots on the cut surface after having been incubated at room temperature at high humidity. Films prepared from the discolored areas showed the presence of large numbers of lactobacilli in apparently pure culture. Films prepared from the normally colored areas showed the presence of large numbers of micrococci, with only an occasional gram-positive rod. When droplets of 3 per cent hydrogen peroxide were applied, the discolored areas appeared to be devoid of catalase, whereas the normally colored areas gave a strong catalase test. The catalase was undoubtedly of microbial origin.

Catalase appears to protect sausages from discoloration due to microbial growth. In one experiment, freshly cut surfaces of steamed wieners were coated with a catalase concentrate of high activity. The surfaces were inoculated with a greening *Lactobacillus* and incubated for 24 hours. No discoloration took place

although extensive growth of the inoculum occurred. Inoculated control samples discolored within 16 hours. Higher dilutions of catalase failed to protect the sausage color since the enzyme appeared to be inactivated at a rapid rate.

It is not implied that the two groups of microorganisms described in this article are the only ones that are capable of causing surface discoloration of sausages. It would seem that any salt-tolerant, catalase-negative microorganism that is capable of growing at low temperatures, and that oxidizes certain substrates with the accumulation of hydrogen peroxide, might be found associated with natural outbreaks of this type of spoilage. Indeed, among the cultures tested one unidentified homofermentative *Lactobacillus* isolated from raw sausage mix was found which discolored sausages in pure culture. From other samples of raw sausage mix, four micrococcus strains were isolated which discolored sausages. The cells of these cultures existed in the form of tetrads. Fitting into the general pattern of the other sausage-greening microorganisms, three of these strains appeared to be catalase-negative under the conditions tested; the fourth strain exhibited extremely weak catalase activity.

More recently, six *Lactobacillus* strains that would discolor sausages in pure culture have been isolated from three samples of boiled ham showing greenish discoloration on the sliced surface. They appeared to be closely related to the greening lactobacilli described, but differed in that they hydrolyzed esculin and arginine and failed to ferment fructose. Of all the substances tested, only glucose, mannose, maltose, and trehalose were fermented by these strains. Unfortunately, five of the six strains were lost before any serological studies were begun. The sixth, however, failed to give a positive precipitin test with the type-specific *Lactobacillus* serum. It therefore appears that these strains represent a second variety of sausage-greening lactobacilli.

All the sausage-greening microorganisms also produced a strong greening reaction on blood agar. This characteristic is not necessarily indicative of their ability to discolor sausages, however. Other than the microorganisms already described, no other "viridans" type of lactic acid bacteria have been found which would promptly discolor sausages, even though they would grow extensively on this product.

The sausage spoilage described in this article may reach "epidemic" proportions and result in serious economic loss if proper precautions are not taken. For control purposes it would be interesting to learn the original habitat of the causative microorganisms. Thus far no light has been thrown on this question.

Although these microorganisms appear to be hitherto undescribed varieties, or species, within their respective genera, the authors hesitate to suggest any new names until our knowledge concerning the classification of the entire group of heterofermentative lactic acid bacteria is less confused.

Vita-Zyme Laboratories, Inc., Chicago, for the generous supply of catalase concentrate.

SUMMARY

A detailed study has been conducted on 30 cultures isolated from outbreaks of greenish discolorations on the surface of sausages. Twenty of the cultures comprised a homogeneous and distinct group of heterofermentative lactobacilli that belonged to one serological type.

The remaining 10 cultures were members of the genus *Leuconostoc*, but these cultures failed to produce mucoid colonies on sucrose agar, although this sugar was fermented.

All sausage-greening cultures were salt-tolerant and capable of growing at low temperatures. Evidence is presented which indicates that the sausage discoloration results from the production of hydrogen peroxide by the microorganisms.

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CHEMICAL DETOXIFICATION OF FLEXNER DYSENTERY ANTIGEN

III. A Mouse Test to Measure Toxicity Relative to Antigenicity[1]

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The problem of developing a detoxified Flexner dysentery antigen for use in the armed forces during the recent war (Barnes, Dewey, Henry, and Lupfer, 1947; Barnes and Dewey, 1947) required a test by which the results of a given chemical or physical manipulation of the antigen could be evaluated with respect to both toxicity and protection. It was necessary to do this with such precision and definition of standards that progress in detoxification could be followed quantitatively. This meant that altered antigens had to be compared with crude vaccines in terms of a smaller or a larger number of toxic units that were capable of yielding an identical degree of protection against infection. To accomplish these ends, it was necessary to adapt several methods to one test and to make this applicable to the dysentery organism. No one test or combination of tests fulfilling these needs was found available or easily adaptable.

In the first place, a representative measure of toxicity of successive dilutions of antigen had to be worked out for the dysentery organism. Although weight loss has been used for many tests of physiological effect, it has not been shown whether it is a generally reliable index of toxicity and it had not been clearly applied to Flexner dysentery toxicity in the mouse as a well-defined test. This convenient property of weight loss was chosen because it was found to be proportional to the logarithm of the dose of antigen given (see below), and in all tests with either unaltered or detoxified material, no discrepancy between weight loss and observed symptoms in mice could be noted.

Secondly, it was important to develop corresponding precision in a protection test rather than by immune body titration because high protectivity against infection was the chief and practical aim; a number of steps had to be taken to achieve adequate precision in this part of the test. For purposes of exploration, the mouse was employed rather than humans, although final results have been checked in the latter (Barnes, Dewey, Henry, and Lupfer, 1947). The protective power of an antigen is titrated a week after its administration by injecting each of the same mice with a carefully standardized dose of live virulent organisms.

Thirdly, it was felt necessary to use the same mice for assaying both toxicity and protection of a given sample; by choice of the tests for weight loss and protection and by adjustment of dosages it was found possible to accomplish this,

although not without considerable difficulty and many trials. The advantages of using the same mice are twofold: (1) unknown secondary effects upon protection from various degrees of toxicity damage to the organism receiving the vaccine are of vital importance and can only be taken into account by using the same mice for the two parts of the test; (2) the errors involved in biological tests are greatly decreased if one set of animals is used to obtain a final value.

A batch of 50 white mice, weighing 15 to 18 g each, are caged in groups of 5 and kept at a constant temperature of 25 C. After 2 days they are weighed to 0.1 g in groups of 5, and each mouse is immediately injected intraperitoneally with 0.5 ml of the vaccine to be tested. The vaccine is diluted so that each suc-

TABLE 1

A toxicity-protection test to demonstrate data

Standard vaccine I—test F XIX

Million organisms per 0.5 ml	64	32	16	8	4	2	1	0.5	0.25	0.125
Weight change in grams	−0.6	0	−0.3	−0.7	+0.2	+0.3	−0.9	0	+0.3	+1.6
	−1.3	−0.8	−0.3	−0.4	−0.3	−0.1	+0.4	−0.1	+0.8	+0.7
	+0.1	−0.1	+0.1	+0.1	+0.2	+0.4	+0.2	+0.6	+0.1	+0.5
	+0.2	−1.0	+0.1	−0.2	+0.1	+0.5	+0.7	+0.3	+1.2	−0.1
	−1.4	−0.9	−0.8	−0.1	+0.3	−0.2	+0.7	+1.0	+0.8	+0.6
	−1.4	−0.7	−0.5	−0.5	+1.0	+0.2	0	+0.1	+0.1	+0.6
	+0.6	0	−0.5	+0.5	−0.7	+0.3	+0.3	−0.1	+0.5	+0.2
	−0.7	−1.2	+0.8	+0.6	−0.1	+0.8	+1.2	−0.2	0	+0.5
	−1.2	−0.3	+0.4	−0.5	+0.5	+0.8	+0.2	+0.3	+0.3	+1.2
	−0.4	−0.1	+0.4	−0.1	+0.6	+0.1	+0.2	+0.5	+0.7	+0.3
Group weight change in grams	−6.1	−5.1	−0.6	−1.3	+1.8	+3.1	+3.0	+2.4	+4.8	+6.1
Protection in no. of mice										
Total	10	10	10	10	10	10	10	10	10	10
Dead	1	1	3	4	4	4	7	10	9	9
Sick and dead	2	2	4	5	6	5	9	10	9	10

cessive concentration is one-half that of the preceding one. Each group of 5 mice receives one of these dilutions. The mice are weighed again in the same order exactly 24 hours after the first weighing. Except for feeding and changing water once during the 24 hours, they are not disturbed in any way during this interval.

Since those groups of mice that receive the largest quantities of antigen generally lose weight and those receiving the smallest amounts generally gain (table 1), the amount of toxin-antigen that brings about no gain or loss in weight may be determined by plotting dilutions against weight loss. This may be done with ordinary unit co-ordinates or, as in figure 1, a straight line may be drawn through the points if dilutions are plotted on a logarithmic co-ordinate. If the highest concentration (zero dilution) is placed at the base line and each 2-fold dilution at

succeeding points along the logarithmic co-ordinate, we find we have plotted values of log $(1 + n)$, in which n is implicit in the expression:

$$C_n = C_o x^{-n}$$

Here C_n and C_o represent concentration at any dilution n and at n equal to zero, respectively, n being a whole number in the series $0, 1, 2, 3, 4, \cdots$ defining the number of 2-fold dilutions made at any point. The dilution factor, x, in all cases described in this paper is equal to 2. An improvement on the straight line is to fit

CHART I
ESTIMATION OF TOXICITY
FRACTION 9,S₁, SERIES XXI
TOXICITY END POINT—22 4
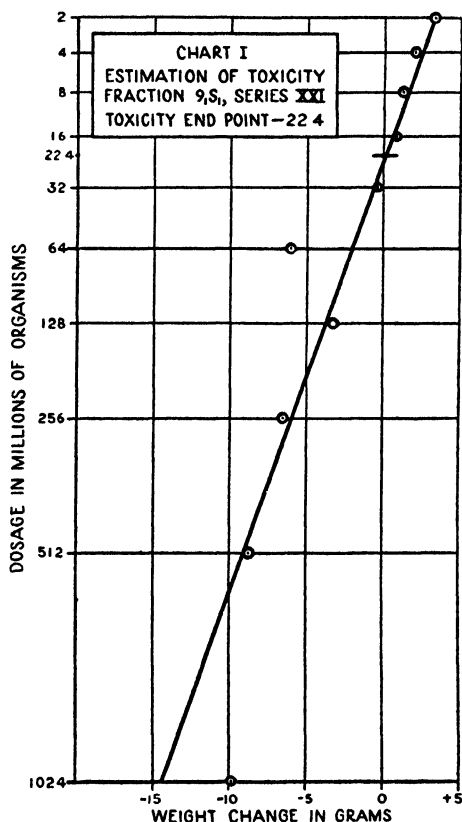
Figure 1.

to the points visually a curved line, the ends of which are made to approach parallelism to the y axis. This alteration at either end is in keeping with the presence of a physiological limit on weight gain and weight loss in any 24-hour period.

If desired, some other end point than zero weight change may be chosen; in fact, we have found that the point of 2-g weight loss for 5 mice is more reliable in comparing some fractions with one another.

Besides graphical methods, another, which permits statistical analysis of the precision in a single test, is to weigh the mice individually. From the series of

records (table 1) one can compute a 50 per cent end point that is the best available estimate of the amount of antigen required to produce a loss of weight in one-half of all mice (e.g., Reed and Muench, 1938; Bliss, 1938).

Seven days after the injection of the vaccine preparation, each mouse is given 0.5 ml of a 3 per cent suspension of mucin containing $\frac{1}{10}$ volume of a 21-hour-old culture of virulent *Shigella paradysenteriae* organisms (about 70 million organisms per mouse). This is 10,000 to 100,000 MLD. To avoid the injection of particles, the mucin suspension is passed through a Waring blender and, after sterilizing, through a hand homogenizer. It is then centrifuged, and to the decanted, neutralized supernatant, which is surrounded by an ice bath and mechanically stirred, the bacteria are added. Aliquots of this suspension are removed by an automatic syringe and injected. Sixteen to 18 hours later the number of sick and dead mice in each cage is recorded. Those mice are counted as sick whose eyes are partially or completely closed by exudate or edema. This end point was chosen because by trial the results were more dependable than by the survival criterion alone. The latter may be subject to many subtle, unknown influences in addition to those involved in the production of a given intensity of diseased state. Exudate and eye closing in this Flexner infection in mice was an objective, unequivocal finding and occurred almost invariably when mice were otherwise distinctly ill. The end point is determined similarly to an LD_{50} end point (Reed and Muench, 1938), as shown in table 1. Also it may be estimated by graphing the results, for which a simple sigmoid curve is given in figure 2.

Each antigen dilution may be recorded as the number of bacteria in millions, or as the number of mg of dried antigen, or as the number of mg of nitrogen to which it corresponds. The level of toxicity of a given antigen divided by the level of protection is the ratio of toxicity to antigenicity for that fraction. This ratio may be compared to that of other fractions titrated simultaneously with other mice, and it is then possible to say that one antigen is so many times more or less effective than another as an immunizing agent. Of two antigens with the ratios expressed as above, that with the higher ratio is the more favorable, i.e., more antigenic for a given degree of toxicity. Finally, a vaccine preparation titrated at one time may be compared with that assayed at another if a standard vaccine is also titrated to provide a point of reference. For this purpose a standard reference vaccine, consisting of killed organisms suspended in saline and dried from the frozen state, was prepared in large amounts.

The test also may be used as a quantitative measure of the proportion of toxicity or of protective power in a chemically prepared fraction of bacteria relative to that present in the whole organism. This is done by comparing the titration end point of a given fraction, in terms of numbers of grams of unaltered bacteria giving that same end point, with the end point obtained by titration of the whole bacteria. Thus, after a given chemical step yielding two products, the fraction of effective antigen in each may be estimated.

The precision of the test can be improved by the use of 10 mice per dilution instead of 5. The reproducibility of the toxicity-protection ratio of a given preparation in a single test using 5-mouse groups is shown in table 2, and the
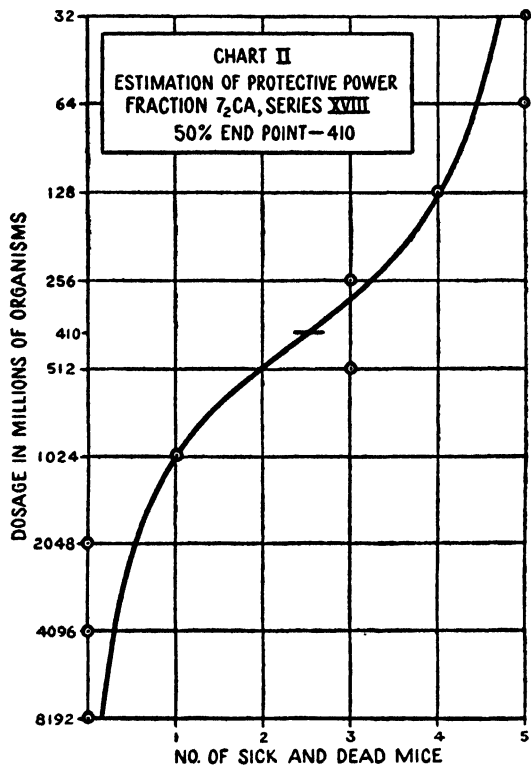
Figure 2:

TABLE 2

Reproducibility of a single test of toxicity-protection ratio

Each vertical column represents a series of tests performed at the same time on various preparations. As the preparations evaluated in a given vertical column are composed of practically identical material, comparison of figures in any one vertical column indicates the reproducibility of estimating an unknown ratio in a given test.

	CODE NO. OF PREPARATIONS TESTED AT A SINGLE TIME			
	No. 19	No. 24	No. 25	No. 26
Standard vaccine no. 1	1.0			
Standard vaccine no. 2	1.4			
Vaccines:				
9H		0.40	0.25	
9_2CS_1		0.45		
$9_2CS_1P_1$		0.70		
$9_2CS_1P_2$.		0.30		
$9_2CS_2P_2$ . . . . :			0.17	
$9_2CS_2P_3$			0.20	
$9_2CS_2P_2$ intraperitoneally				2.7
$9_2CS_2P_2$ subcutaneously				2.2

reproducibility of the comparison between two fractions made in different test runs is shown in table 3. More data on reproducibility were not obtained because of the need for applying the test as much and as quickly as possible to the development of a needed vaccine, but 150 separate test runs in the course of the work (Barnes, Carroll, Henry, and Lupfer, to be published) gave useful, dependable results (for example, see table 4). It should be emphasized that it is

TABLE 3

Reproducibility of the ratio of a toxicity-protection value to that of a standard value

The figures represent $\frac{Ru}{Rs}$ in which Ru equals the toxicity-protection ratio for an unknown fraction on a given test and Rs the ratio for the standard vaccine on the same test. Comparison of values in a horizontal column indicates the reproducibility of $\frac{Ru}{Rs}$ for a single fraction from week to week in different test runs.

	CODE NO. OF TESTS MADE AT A SINGLE TIME		
	No. 22	No. 24	No. 25
Vaccines:			
9H......	0.75		0.60
$9_3CS_2P_2$.		0.55	0.70

TABLE 4

Results of various conditions of storage on some antigen fractions

Values represent percentage of detoxification, D; see text. Five hundred and seventy mice were used to perform the screening tests listed here.

	DURATION OF STORAGE—WEEKS												TYPE OF MOUSE TEST USED
	0	¼	½	1	1½	3	4	13	14	21	25	33	
Vaccines													
$9_3S_2P_2$................. ..	0					14	80	84	99	91			50-mouse titration, 5 mice per dilution
Standard vaccine II......	0						76				88	97	50-mouse titration, 5 mice per dilution
Whole organisms, dried, 0 C....................	0	58	67	75	34	75							20-mouse screening test
			58			71							
$10S_2P_3$....................	0	61		54									10-mouse screening test
				66									

important to pick healthy mice, to maintain hygienic conditions for the mice, and to follow carefully all details here outlined.

When a screening method is needed for survey observations, the test may be made in an abbreviated form. We have used it extensively in this manner and have found it a useful index of progress in surveying a large number of antigens. The abbreviated form is conducted in the following way:

(a) To 20 mice, weighed in groups of 5, antigen treated for the purpose of de-

toxifying it is given in dosage, A_t, so chosen that the untreated, undetoxified material in the same dosage gives a weight loss in 10 mice of about 20 to 22 g in 24 hours.
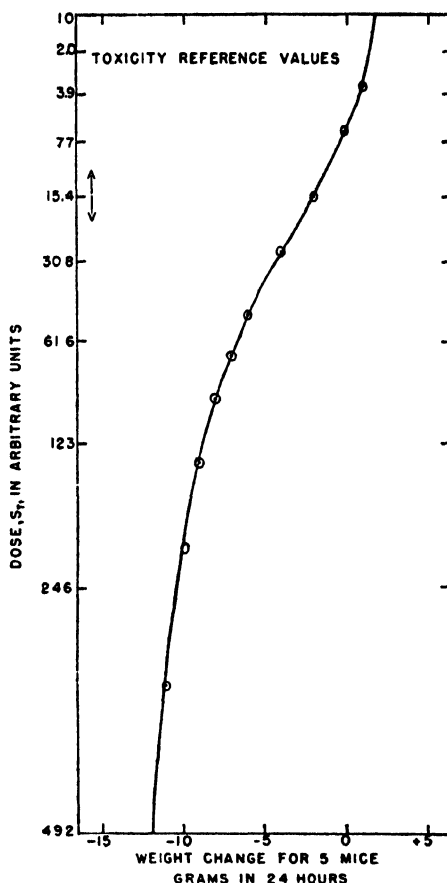
Figure 3. Average toxicity of eleven different untreated antigen preparations.

In order to provide a common unit basis for each of the 11 preparations, the point of 2-g weight loss in 5 mice was arbitrarily assigned the same dosage value in each case, and the other points of the y axis were adjusted accordingly so as to relate the dilutions by a factor of 2. As this arbitrary level could be set at any figure, it was so chosen in relation to the unitage for the protection curve (q.v.) that the average toxicity-protection ratio from 8 different measurements of unaltered antigen at the 100-μg level was equal to one (see equations in text). The dosage so chosen was 15.4 μg at the level of 2-g weight loss in 5 mice. The standard deviations of the dosage units for each point of the graph are (in order from the highest dosage): 82.3, 70.2, 32.9, 20.5, 13.5, 5.50, 1.08, zero, 1.11 (dosage at $+1$ = g weight change not used).

(b) A number of such treated preparations may be given during the same week to different batches of 20 mice and thus many possibilities may be explored at once.

(c) Each week a standard vaccine, kept in dry form at 4 C, is given in the same weekly dosage, W_t.

(d) From a previously prepared graph relating toxicity to dose of untreated antigen and compiled from averages of a number of mouse titrations performed
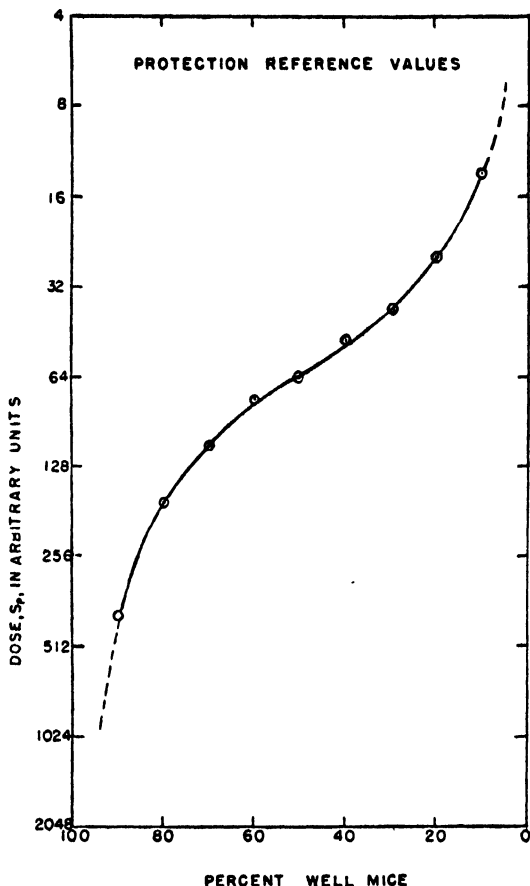
Figure 4. Average protection of eight different untreated antigen preparations.

In order to provide a common unit basis for each of the 8 preparations, the 50 per cent end point was arbitrarily assigned the same dosage value in each case and the other points on the y axis were adjusted accordingly so as to relate dilutions by a factor of 2. This value was set at 64 because of the convenience of this number for expressing related concentrations by a factor of 2. The standard deviations of the dosage units for each point on the graph are (in order from the highest dosage): 107.3, 28.0, 6.63, 4.72, zero, 4.32, 4.59, 5.78, 3.52.

on unaltered antigen (see figure 3) the weight loss incurred by the mice for a given preparation or standard is read in terms of equivalent dose, S_{ta}, S_{tw}, respectively, of unaltered reference vaccine (see legend, figure 3).

(e) Protection tests are run simultaneously on all batches the next week, in the manner previously described, and from a standard graph, similarly pre-

pared from averages, the percentage of protection *found* is read in terms of dosage, S_{pa}, S_{pw}, of unaltered reference vaccine (figure 4).

(f) The doses of treated antigen and of weekly standard *given* to the mice in this protection portion of the test are referred to as A_p and W_p, respectively. (Usually these refer to the same material that was given the week before to test toxicity, but the protection part of the procedure may be tested separately at some different dosage level if desired.)

It can be shown readily that the percentage of decrease in toxicity, D, of a given preparation of antigen, A, is expressed by the following:

$$D = 100 - \frac{(100\,S_{ta}}{(\quad A_t} \times \frac{W_t)}{S_{tw}} \frac{(A_p}{(S_{pa}} \times \frac{S_{pw})}{W_p)}$$

However, in all of our tests, the doses given to estimate toxicity and protection are the same for any given preparation, A'; therefore $A'_p = A'_t$ and $W_p = W_t$. It follows that:

$$D = 100 - \frac{(100\,S_{ta}')}{(\quad S_{pa}'\quad)} \frac{(S_{pw})}{(S_{tw})}$$

Since the ratio $\dfrac{S_{pw}}{S_{tw}}$ was not considered significantly altered from one (see legend, figure 3), the expression usually could be used in a shortened form as follows:

$$D = 100 - \frac{(100\,S_{ta}')}{(\quad S_{pa}'\quad)}$$

Thus D expresses complete loss of toxicity as 100 per cent detoxification; or, if the toxicity of a given dose of treated antigen were reduced to that obtained from ¼ dose of unaltered material, D would equal 75 per cent.

The error in terms of standard deviations for the reference curves is given in the legends to figures 3 and 4. The reproducibility of the 20-mouse screening test is indicated in table 4, in which the results of successive tests over many weeks show a consistent loss of net toxicity for all but the last fraction, which was not appreciably changed.

SUMMARY

A method for measurement of changes in toxicity relative to antigenicity of a vaccine is described. The test uses a single series of mice for the assay of both quantities, toxicity being estimated by weight loss, and protection, after a week's interval, by resistance to a challenging dose of live organisms.

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A PURINE-REQUIRING STRAIN OF PHOTOBACTERIUM FISCHERI

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The production of mutant strains of *Photobacterium fischeri* has been reported by McElroy and Farghaly (1948), who succeeded in isolating amino acid mutants following exposure to ultraviolet radiations. The examination of such mutants as these may prove to be of value in the study of the relationships between respiratory and luminescent systems within the cells.

In the course of studies upon the respiration of *Photobacterium fischeri* it was necessary occasionally to reisolate in order to maintain a pure culture. This was accomplished by the conventional plating method. Brightly glowing colonies picked from the agar surface were transferred to slants of a complete medium for use as stock cultures. This report deals with a purine-requiring mutant strain of this organism which was isolated in the foregoing manner from the parent culture.[1]

The failure of a basal medium described and used by Doudoroff (1942) to support growth of this organism,[2] led to the investigation of its nutritional requirements. It was discovered that growth occurred only in the presence of one of the following purine bases: adenine, hypoxanthine, guanine, or xanthine. The extent of growth was found to be proportional to the amount of purine present.

There are descriptions in the literature of purines that may function as essential growth factors or stimulatory substances for particular organisms. Guthrie (1949) has described an *Escherichia coli* mutant that possesses purine requirements quite similar to those of the *P. fischeri* mutant described here. Tatum (1946) produced a mutant strain of *E. coli* which required purines, and Pappenheimer and Hottle (1940) found a strain of hemolytic streptococcus with a purine requirement. Mitchell and Houlahan (1946) produced a *Neurospora* mutant deficient for both adenine and hypoxanthine, and these investigators have proposed this mutant for use as an assay organism.

MATERIALS AND METHODS

As noted above, the strain used in these studies was a naturally occurring spontaneous mutant isolated from poured plates that were used as sources for pure cultures. These bacteria were maintained on agar slants of the following

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[1] The original culture was obtained from a commercial source, the Carolina Biological Supply Co., Inc., Elon College, North Carolina.

[2] Doudoroff's medium consisted of distilled water containing M/30 Sørensen's KH$_2$PO$_4$-Na$_2$HPO$_4$ buffer at pH 7.0; 3 per cent NaCl; 0.03 per cent NH$_4$Cl; 0.03 per cent MgSO$_4$; 0.001 per cent FeCl$_3$; 0.001 per cent CaCl$_2$, and 0.1 to 0.3 per cent organic carbon source by weight.

composition per liter of medium: Difco nutrient agar, 30 g; NaCl, 30 g; K_2HPO_4, 1 g; and $MgSO_4 \cdot 7H_2O$, 0.5 g.

The organisms were grown at room temperature (23 to 26 C) for 24 hours and were then stored in a refrigerator. For preparation of the inoculum, the organisms were grown for 24 hours at room temperature in broth of the following composition: Difco peptone, 30 g; NaCl, 30 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; and distilled water, 1 liter.

The inoculum was prepared by transferring cells from a 20- to 24-hour slant to a tube of broth. This was incubated for 12 to 18 hours, and then centrifuged and decanted. The cells were washed twice with 10 ml of sterile 0.5 M NaCl and were finally suspended in 10 ml of sterile saline. The inoculum usually gave a turbidity reading of 15 to 25 on a Klett-Summerson photoelectric colorimeter. One drop of this inoculum was pipetted aseptically into each tube of medium.

Basal medium. All experiments were performed in a basal medium of the following composition:

10% vitamin-free casein hydrolyzate	10 ml
NaCl	30 g
K_2HPO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$CaCl_2$	0.1 g
Mineral supplements[3]	
Vitamin supplement[4]	
Tryptophan	30 mg
Cystine	30 mg
Glucose[5]	10 g
Distilled water	1,000 ml

All growth experiments were performed in pyrex test tubes of 13-by-100-mm size, which were selected to agree within 3 units on the scale of a Klett-Summerson photoelectric colorimeter using a no. 42 filter. The basal medium, which was concentrated proportionally, was dispensed in 3.0-ml quantities into these test tubes. Water, glucose, and the supplements that were to be tested were added to make a final volume of 5.0 ml. All tubes for these tests were prepared in triplicate. The tubes were sterilized by autoclaving for 15 minutes at 15 pounds pressure. Following inoculation, the tubes were incubated at room temperature (23 to 25 C). Results were read in the photoelectric colorimeter at varying intervals, depending upon the type of experiment being performed, but growth was usually not at a maximum level until 72 hours of incubation.

[3] The following minor mineral elements were added in parts per million: 0.005 B; 0.01 Mn, 0.09 Zn; 0.02 Cu, 0.01 Mo, and 0.1 Fe.

[4] The vitamin supplement was added so that the final concentration per ml became: thiamine: HCl, 1 mμ mole; pyridoxine: HCl, 1 mμ mole; niacin, 1 mμ mole; biotin methyl ester, 0.0001 μg; calcium pantothenate, 1 mμ mole; riboflavin, 1 mμ mole; and pteroylglutamic acid, 1 mμ mole.

[5] It was found, in accordance with the observations of Doudoroff (1942), that growth would occur in this medium only if the glucose was sterilized and added separately. Accordingly, 1 ml of sterile glucose was added aseptically to each tube of autoclaved medium to give a final concentration of 10 mg per ml of medium.

RESULTS

In view of the fact that the purine requirement was relatively nonspecific, several closely related compounds were also studied as possible substitutes for the purine bases. Guanosine (guanine-riboside), guanylic acid (guanyl-ribo-furano-3-phosphoric acid), and ribonucleic acid were all found to replace the purines completely. On the other hand, it was discovered that arginine, histidine, caffeine, uric acid, thymine, and uracil were completely inactive. With the exception of histidine and arginine, these substances were tested at a concentration of 10 μg per ml. Histidine and arginine were added at a concentration of 1.0 mg per ml.

By the use of varied amounts of purines a graded growth response was obtained which produced curves of growth response quite similar to the standard
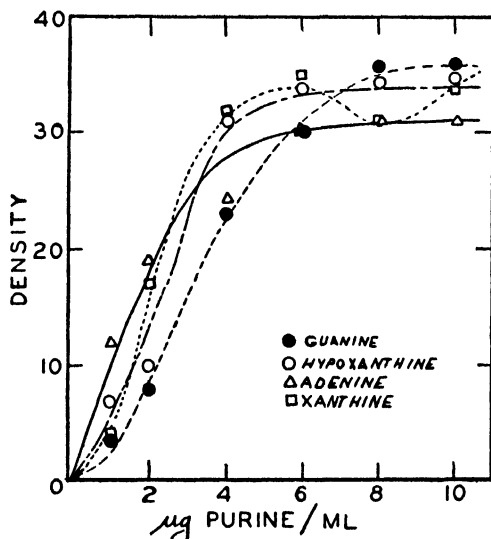
Figure 1. Growth response of mutant strain to purines.

assay curves of other organisms. Amounts of purines ranging from 1.0 to 10.0 μg per ml of medium were used. Turbidity readings were recorded at 72 hours. Typical growth curves were obtained, as indicated in figure 1. As might be anticipated, the growth responses were generally but not precisely reproducible. The small differences are not especially significant since they can be accounted for by slight variations in the medium, the extent of aeration, the temperature of incubation, and the inoculum.

Luminescence. In this instance the extent of bioluminescence was not of particular concern. No quantitative and but few qualitative measurements were made. There was, however, a minimum of luminescence observed in all cases. As the cultures were not aerated in any special way, this low level of brightness indicates a minimum oxygen supply, since 2.0 mm Hg partial pressure of oxygen is required for maximum luminescence in these organisms (Shoup, 1929).

DISCUSSION

Since relatively large quantities of purines are required for growth, it is probable that the purines do not function as enzymes or as coenzymes but are used by this organism in the synthesis of certain essential protoplasmic units. Nishimura (1893), Long (1921), Coghill (1931), and others have demonstrated the presence of adenine and guanine in the nucleic acids of bacteria. Since adenine and guanine are of such frequent occurrence as components of nucleic acids, it is possible that these purines are utilized by the organism in the synthesis of nucleic acids and related substances. It is not known whether the purines are incorporated directly into the proteinaceous material or are subjected to previous modification.

The role of xanthine and hypoxanthine is not clear. These purines are doubtful components of nucleic acids, although they have been isolated from bacterial cells by Stoklasa (1908), Tamura (1913), and Aronson (1900). Guanine and adenine are generally considered the only naturally occurring purine bases in nucleic acids, and the oxypurines, xanthine and hypoxanthine, may be derived from these by enzyme action (Porter, 1946). The enzyme guanase catalyzes the deamination of guanine to produce xanthine and ammonia, and the enzyme adenase catalyzes the deamination of adenine to produce hypoxanthine and ammonia. The fact that the four purines are so closely related structurally may lead one to suspect that only one of the group is actually the growth factor and the other three are enzymatically converted to that compound by the bacteria.

ACKNOWLEDGMENT

SUMMARY

A new strain of *Photobacterium fischeri*, a marine form that requires one of the following purines for growth, xanthine, guanine, adenine, or hypoxanthine, has been described. These purines may be replaced by guanosine or guanylic acid, but cannot be replaced by caffeine, uric acid, arginine, histidine, thymine, or uracil. Growth response curves of single purines roughly parallel one another. The possible role of the purines in the metabolism of the organism is discussed.

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A STREPTOMYCETE PATHOGENIC TO FISH

ROBERT R. RUCKER

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A streptomycete and pseudomonad were isolated from blueback salmon, *Oncorhynchus nerka* (Walbaum), and shown to be pathogenic to fish. These organisms were isolated from young blueback salmon taken from a group that developed an increasing mortality after feeding about a month at the United States Fishery Station, Leavenworth, Washington. A superficial examination revealed only the presence of fungus (probably *Saprolegnia parasitica*), which was on the gills and was eliminated by treatment with a quaternary ammonium salt. Although the fungus infection was eliminated, the mortality continued.

It was observed by the station biologist at the time that the majority of the fish in the hatchery troughs were healthy, but that there was always present an apathetic group that huddled on the bottom, refused food, and eventually weakened and died. The bulk of the daily mortality was composed of fish from this group. The apathetic group received constant recruitment from the more vigorous stock, and their number showed a gradual increase rather than depletion. A more critical examination of the larger affected fish revealed that the kidneys and spleens were disintegrating, mycelial masses were sporadically observed in the body cavity, congestion was present in the gastrointestinal tract, some hemorrhagic areas were present in the body musculature, and a few fish had a perforating ulceration of the body wall.

Furunculosis was immediately suspected, and attempts were made to isolate from the diseased fish *Bacterium salmonicida* Lehmann and Neumann, the etiological agent of furunculosis. *B. salmonicida* was not recovered, however, even after repeated attempts at isolation. Subsequently it was discovered that two other organisms, a streptomycete and a pseudomonad, were characteristically present in the diseased fish. Both organisms were found experimentally to be pathogenic to fish.

MATERIALS AND METHODS

Material from the spleens and kidneys of diseased fish was streaked on 1 per cent agar plates containing 0.5 per cent tryptone and adjusted to pH 7.2. After incubation for 2 to 3 days at 20 C, one or more of each of the different types of colonies appearing on the plates were picked and incubated in tryptone broth. Blueback salmon fingerlings were inoculated subcutaneously, intramuscularly, or intraperitoneally with these cultures. Organisms causing a pathological effect culminating in death were recovered and investigated.[1]

Cultures were taken from infected fish at the Leavenworth Station on four

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[1] Facilities for the experimental retention of fish were furnished by the School of Fisheries, University of Washington.

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different occasions over a two-month period. The cultures first isolated (March 19, 1947) were limited to those resembling *B. salmonicida*; no pathogens were found. A greater variety of cultures was tested from the isolates obtained on April 2. A *Pseudomonas* sp. was isolated and shown to be pathogenic at this time. On April 22 the *Pseudomonas* sp. was again isolated from a spleen dilution series. Also on this date a pathogenic streptomycete was isolated from 9 of 20 kidney streaks. The streptomycete presumably was not recognized prior to this time because the plates were not incubated for a sufficient length of time. The last cultures taken from the group of fish, May 15, showed both the pseudomonad and the streptomycete to be present.
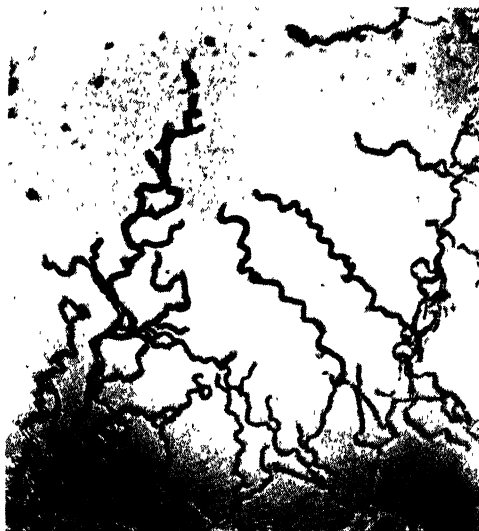
Figure 1. Aerial hyphae of *Streptomyces salmonicida.* × 1,000.

RESULTS AND DISCUSSION

The two organisms, a streptomycete and a pseudomonad, having been found pathogenic to fish, were further investigated. The pseudomonad was shown to be similar to *Pseudomonas punctata* (Zimmerman) Chester, but future investigations may show it to be a new species. It will not be discussed further in this report, which will be limited to the pathogenic streptomycete. It is strange that no type of actinomycosis has been reported previously from fish, as actinomycoses of humans and animals are known to be ubiquitous.

Identification of streptomycete. Nodules containing a tangled mycelial mass were formed in fish, either from a natural infection or artificial inoculation. The hyphae were 0.5 μ to 1 μ in diameter, branched, nonseptate, gram-positive, and not acid-fast. On the agar medium the hyphae were 0.5 μ to 0.75 μ in diameter, the aerial forms were long open spirals that developed oval conidia (0.6 μ by 1.5 μ) in chains (figure 1). The colony on agar was at times smooth and about 1 mm in diameter (figure 2); yet at other times it was larger, up to 3 mm in diam-

eter. The larger colonies (figure 3) were conical in cross section, had an elevated central papilla, and showed marked radial foldings. The submerged mycelium was somewhat limited. As conidia developed, the surface of the colony developed a white or pink-white, powdery coat, at which time a musty or earthy odor was manifest. On tryptone broth a heavy, white, tenacious pellicle was formed. Growth on potato after 3 days was scanty, beaded, gray, and dry; after 10 days it was heavy, beaded, pinkish gray, and dry. No effect was produced on the potato.

No acid or visible gas was produced from glucose, lactose, sucrose, maltose, or mannitol in tryptone broth; litmus milk was unchanged; gelatin was not liquefied; starch was not hydrolyzed; and indole was not formed (Ehrlich's
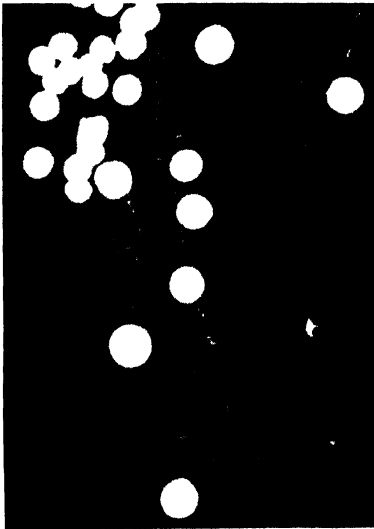
Figure 2   Figure 3

Figure 2. Small type of agar colony, S. salmonicida, 10 days old. × 4
Figure 3. Large, typical actinomycete agar colony, S. salmonicida, 10 days old × 4
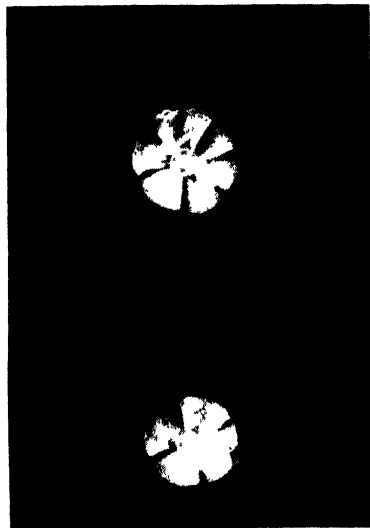
reagent). Nitrites were produced from nitrates, and hydrogen sulfide was produced in tryptone broth as revealed by the lead acetate, filter paper method. A brown, water-soluble pigment developed in agar. All of these tests were conducted at room temperature or 20 C. The effect of temperature on the growth of the organism was as follows: fair at 20 C, good at 30 C, and no growth at 37 C. The organism is aerobic and will not grow anaerobically.

Following the key to the genera of the family Streptomycetaceae proposed by Waksman and Henrici (1943) and adopted, with modifications, in Bergey's Manual (Breed et al., 1948), this species should be placed in the genus Streptomyces and in group 3 on the basis of the structure of the sporulating hyphae. A new specific name, Streptomyces salmonicida n.sp., is suggested for this organism, which is the first in this family to be described as a fish pathogen.

Pathogenicity. The pathogenicity of a natural infection by the two isolated

organisms can be evaluated only from the hatchery mortality records. The natural pathogenicity of each organism could not be determined because both organisms were occasionally present in fish simultaneously. The mortality records (table 1) during January (3.5 per cent) reveal only the normal loss anticipated during the transitional period when the yolk material is being absorbed and the fish are learning to eat. It is assumed, therefore, that the disease, if present during January, did not contribute to the recorded mortality. The increased mortality during February (4.0 per cent), March (7.0 per cent), and April (8.0 per cent) was not normal and was attributed to the disease. The losses continued to increase and were highest in June (30 per cent), after which time the hatcherymen disposed of the diseased fish. The average water temperatures remained in the mid-forties until the latter part of June, when the temperatures approached 50 F.

The pathogenicity in an induced infection by *Streptomyces salmonicida* was determined by inoculating healthy blueback salmon fingerlings under experimental conditions. The results of one experiment in which 40 blueback salmon

TABLE 1

Mortality data of blueback salmon stock at the Leavenworth hatchery during the 1947 season

DATE	MORTALITY	DATE	MORTALITY
	%		%
January	3 5	April	8.0
February	4.0	May	20.0
March	7.0	June	30.0

fingerlings were injected either intraperitoneally or intramuscularly were as follows: the fish started to die about a week after inoculation; there was an increase in mortality about the twelfth day and then the losses were 1 or 2 a day for 2 weeks. After a total of 64 days, all but 3 of the fish had died with the typical symptoms of the disease. Fish fed the organism, and the unexposed controls, showed no loss for the 64-day period.

Pathology. The pathology of natural infections by each of the two organisms could not be determined because of their concurrent appearance, as mentioned earlier. In laboratory-induced infections, however, it was shown that fish inoculated intramuscularly with pure cultures of *S. salmonicida* developed necrosis at the sites of injection. This necrotic area spread slowly, sometimes allowing fungus (*Saprolegnia* sp.) to develop on the lesion, indicating a state of debility, before the fish died. Nodules of the streptomycete were often present in these lesions.

Intraperitoneal injections lead to the development of mycelial masses along the intestine, as shown in figure 4, and the development of a more diffuse growth of the organism on and in various tissues. Figure 5 shows a mycelial mass on the surface of the liver, with the hyphae shown penetrating the organ and causing

an underlying focal necrosis. A most remarkable observation is that, in spite of this, there is apparently no significant inflammatory response on the part of the host.

Figure 4. Nodules of *S salmonicida* on the viscera of an inoculated blueback salmon *Oncorhynchus nerka* × 1 5

Figure 5. Liver of blueback salmon fingerling with *S. salmonicida* on the surface at the left showing an underlying area of focal necrosis Normal tissue is at the right. × 150

Experimental infection caused by the *Pseudomonas* sp. in pure culture, in contrast to the description, given in the introduction, of the symptoms when both organisms were present, is characterized by small areas of erythema on the surface of the fish; peritonitis often develops; and the fish usually die from septicemia.

SUMMARY

A new fish pathogen isolated from diseased blueback salmon fingerlings is described. The organism, named *Streptomyces salmonicida* n.sp., causes a chronic disease terminated by necrosis and death.

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THE TOXICITY OF CERTAIN AMINO ACIDS FOR BRUCELLAE

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In a previous paper (Schuhardt *et al.*, 1949) we suggested the possibility of certain oxidized amino acids being the antibrucella factor in toxic peptones. This possibility is in line with the numerous reported instances of the toxicity of amino acids for bacteria following the pioneer work of McLeod and his colleagues (Wyon and McLeod, 1923; Gordon and McLeod, 1926; McLeod, Wheatley, and Phelon, 1927) and that of Gladstone (1939). The latter report was doubly significant in that it introduced the principle of competitive neutralization of the antibacterial toxicity of one amino acid by other amino acids.

Several workers have reported the growth of *Brucella* spp. on chemically defined media containing amino acids (Koser, Breslove, and Dorfman, 1941; McCullough, Mills, Herbst, Roessler, and Brewer, 1947; Gerhardt and Wilson, 1948). The synthetic media used by the first two groups of workers contained 17 and 14 amino acids, respectively, and conceivably, therefore, might possess both toxic and neutralizing amino acids in their composition. The Gerhardt and Wilson medium contained DL-asparagine as the sole source of nitrogen.

McCullough and Dick (1943), in this laboratory, reported the growth of certain strains of all three *Brucella* species on an amino-acid-free medium containing inorganic ammonium salt as the sole source of nitrogen. None of these synthetic media will grow all strains of brucellae tested, and none of the authors claimed successful growth of freshly isolated, CO_2-requiring strains of *Brucella abortus*. The McCullough and Dick medium, although admittedly deficient for many strains of brucellae, seemed to offer a suitable base medium for testing the toxicity of various amino acids for strains of brucellae that would grow in this unsupplemented base medium.

EXPERIMENTAL RESULTS

The toxicity of casein amino acids for Brucella abortus 1257. Since the synthetic media of Koser *et al.* (1941) and McCullough *et al.* (1947) contained most of the amino acids of casein, and since casein digests are commonly advocated for *Brucella* culture media, we decided to do a preliminary test on the 19 amino acids found in casein combined in the relative concentrations in which they occur in natural casein. The casein amino acids (CAA) were then tested at concentrations of 0.5, 1.0, and 1.5 per cent casein equivalence in the basal medium of McCullough and Dick minus the ammonium salt. The pH of each medium was adjusted to 7.0, and each medium was distributed in 5-ml amounts to duplicate series of 10 tubes each and sterilized at 121 C for 20 minutes. The tubes were

inoculated with 0.1-ml amounts of 10-fold dilutions through 10^{-9} of a 48-hour culture of *Brucella abortus* 1257. The inoculated tubes were incubated at 37 C and examined for growth at 2, 3, and 5 days. Growth as evidenced by developing turbidity was arbitrarily designated 4+, 3+, 2+, 1+, ±, and −. Table 1 lists the results of this experiment.

These results indicate that at 0.5 per cent concentration our casein amino acids serve as an acceptable source of nitrogen with little or no evidence of toxicity for *B. abortus* 1257. When this concentration of amino acids is doubled or trebled, the medium acquires a toxicity very similar to that observed in toxic peptones in that only the largest inocula are able to attain visible growth.

TABLE 1

The toxicity of casein amino acids* for Brucella abortus 1257 as measured by a graded inocula test

INOCULA 0.1 ML.	CONCENTRATIONS OF CASEIN AMINO ACIDS								
	0.5%			1.0%			1.5%		
	Incubation period, days								
	2	3	5	2	3	5	2	3	5
Undil.	3+	3+	4+	1+	1+	2+	±	±	2+
10^{-1}	2+	3+	4+	−	±	1+	−	−	−
10^{-2}	2+	2+	4+	−	−	±	−	−	−
10^{-3}	1+	2+	4+	−	−	−	−	−	−
10^{-4}	±	1+	4+	−	−	−	−	−	−
10^{-5}	−	±	3+	−	−	−	−	−	−
10^{-6}	−	±	3+	−	−	−	−	−	−
10^{-7}	−	−	3+	−	−	−	−	−	−
10^{-8}	−	±	8+	−	−	−	−	−	−
10^{-9}	−	−	3+	−	−	−	−	−	−

* The 19 amino acids of casein combined in distilled water in the relative concentrations found in normal casein.

The only interpretation that we can visualize for these results is that one or more of the amino acids of our CAA is toxic for *B. abortus* 1257 at the concentration present in 1.0 and 1.5 per cent, and that this toxicity is not neutralized by comparable increased concentrations of the other amino acids above those in 0.5 per cent CAA. This observation necessitated the testing of the individual amino acids of this series for toxicity for this organism.

Toxicity of the individual casein amino acids for B. abortus 1257. The 19 amino acids that had been incorporated in the CAA were tested individually for toxicity to *B. abortus* 1257 at concentrations above and below that found in 1 per cent CAA. Tubes containing the various concentrations of the amino acid being tested, in 5 ml of McCullough-Dick medium, were inoculated with 0.1 ml each of a 10^{-3} dilution of a 48-hour broth culture of the test organism. The tubes were incubated at 37 C for 5 days and examined for growth in comparison with inoculated tubes of McCullough-Dick medium containing no amino acid.

Table 2 records the results of this experiment. A greater than (>) sign indicates that no inhibition of growth of the type indicated was observed at the amino acid concentration listed, which was the highest concentration tested. Where inhibition of growth was observed, the smallest concentrations giving partial and complete inhibition are recorded. These results indicate moderate toxicity of phenylalanine and methionine and marked toxicity of tryptophan and cystine for *B. abortus* 1257. As might be expected, some of the amino acids tended to stimulate rather than inhibit growth of this organism.

TABLE 2

Toxicity of individual casein amino acids for Brucella abortus 1257

AMINO ACID	QUANTITY PRESENT IN 1% AMINO ACID CASEIN,* µg PER ML	QUANTITY REQUIRED PARTIALLY TO INHIBIT GROWTH, µg PER ML	QUANTITY REQUIRED TO COMPLETELY INHIBIT GROWTH, µG PER ML
Glutamic acid	2,180	>2,000	>2,000
Proline	800	>1,000	>1,000
Histidine	760	>1,000	>1,000
Arginine	520	>1,000	>1,000
Lysine	260	>1,000	>1,000
Alanine	180	>1,000	>1,000
Glycine	40	1,000	>1,000
Serine	580	500	>1,000
Aspartic acid	410	500	>1,000
Hydroxyproline	20	500	>1,000
Threonine	390	250	>1,000
Tyrosine	650	4	>1,000
Valine	790	500	1,000
Leucine	970	500	1,000
Isoleucine		250	1,000
Methionine	360	6	500
Phenylalanine	390	4	500
Cystine	30	4	8
Tryptophan	220	<4	4

* The values for the amino acid content of casein were taken from R. J. Williams, Textbook of Biochemistry, 2d ed., D. Van Nostrand Co., Inc., New York, 1942.

Competitive neutralization of amino acid toxicity for B. abortus 1257. A series of experiments were conducted to determine which if any of the other amino acids of the CAA would reverse the inhibition of the toxic amino acids for *B. abortus* 1257. The technique employed was essentially the same as that given above except that varying concentrations of other amino acids were added to the tubes containing an inhibitory concentration of the toxic amino acid. When reversal was observed, tests were made to determine whether or not the reversal was competitive over a range of inhibitory concentrations of the toxic compound. A partial summarization of the results of these tests is given in table 3.

Tryptophan toxicity for *B. abortus* 1257 was competitively reversed by phenylalanine, tyrosine, and histidine over a 32-fold range of its individually toxic concentration. Also 400 µg per ml of tryptophan, when added to 1 per cent

casein hydrolyzate and to 1 per cent nontoxic tryptose, failed to render these media toxic for *B. abortus* 1257. Therefore we are convinced that tryptophan is not responsible for the toxicity of 1 per cent CAA nor for the toxicity of peptones for brucellae.

Methionine and phenylalanine toxicities for *B. abortus* 1257 were readily reversed by other amino acids at concentrations of the toxic pair well above those found in 1.0 per cent CAA. Also since they failed to toxify casein hydrolyzates and nontoxic tryptose, they too are eliminated from the probability of being the toxic factor in 1.0 per cent CAA and in toxic peptones.

TABLE 3

Neutralization of the toxicity of methionine, tryptophan, and cystine by other amino acids

INHIBITORY AMINO ACID, μG PER ML		NEUTRALIZING AMINO ACID, μG PER ML REQUIRED		GROWTH, 5 DAYS
Tryptophan	16	None		−
	32	Phenylalanine	10	+
	32	Tyrosine	40	+
	32	Histidine	80	+
	64	Phenylalanine	40	+
	64	Tyrosine	80	+
	64	Histidine	320	+
	128	Phenylalanine	80	+
	128	Tyrosine	160	+
	128	Histidine	640	+
Methionine	500	None		−
	1,000	Glutamic acid	10	+
	1,000	Alanine	1,000	+
	1,000	Lysine	1,000	+
Cystine	4	None		−
	4	Glutamic acid	8	+
	8	Glutamic acid	125	+
	16	Glutamic acid	1,000	+
	32	Glutamic acid	2,000	+
	64	Glutamic acid	2,500	−

Cystine toxicity for this organism, however, was reversed by none of the other 18 amino acids tested with the exception of glutamic acid. This reversal was competitive over a wide range, but in the vicinity of the concentrations of the two amino acids expected in 1.0 per cent casein digests, the glutamic acid neutralization of the cystine toxicity was borderline. Furthermore, the addition of cystine to 1.0 per cent casein hydrolyzate or to 1.0 per cent nontoxic tryptose tended to toxify these media for all but the largest inocula of *B. abortus* 1257.

Thus cystine could be the cause of the toxicity of 1.0 per cent CAA and could be the antibrucella factor in toxic tryptose. To be the latter, however, there would have to be either an excess of cystine, or an insufficiency of glutamic acid, or a counteraction of the cystine neutralizing effect of the glutamic acid by

other compounds present in the medium. That the latter might be a possibility is evidenced by the fact that 2,000 μg per ml of glutamic acid neutralized 32 μg per ml of cystine in the absence of other amino acids (table 3), whereas 2,180 μg per ml of glutamic acid failed to neutralize 30 μg per ml of cystine in the 1.0 per cent CAA experiment (table 1). The repetition of the 1.0 per cent CAA experiment with varying amounts of cystine, however, did not substantiate this concept (table 4). Probably differences in the number of organisms in different 48-hour cultures used in preparing the inocula and possibly other factors play some part in these borderline relationships. These interrelationships of cystine, glutamic acid, and other amino acids are being investigated further in an effort to determine their possible significance in toxic and nontoxic tryptose and other peptones.

TABLE 4

Toxicity of casein amino acids for B. abortus 1257 due to its cystine content

INOCULA	CYSTINE CONCENTRATION—μG PER ML					
	10	20	30	40	50	60
	Growth—5 days					
Undil.	4+	4+	4+	4+	4+	4+
10^{-1}	4+	4+	4+	4+	4+	4+
10^{-2}	4+	4+	4+	4+	−	2+
10^{-3}	4+	4+	4+	2+	−	−
10^{-4}	4+	4+	4+	−	−	−
10^{-5}	4+	4+	4+	−	−	−
10^{-6}	4+	4+	4+	−	−	−
10^{-7}	4+	4+	4+	−	−	−
10^{-8}	4+	3+	2+	−	−	−
10^{-9}	3+	3+	2+	−	−	−

Studies on the toxicity of cystine for brucellae. In an effort to determine whether or not the observed toxicity of our 1.0 and 1.5 per cent CAA was due to the cystine content, we repeated the casein amino acids toxicity experiment using all the other amino acids at 1.0 per cent CAA concentrations but varying the cystine concentration from 10 to 60 μg per ml. Table 4 gives the results of this experiment, which seems to leave little doubt that cystine was the cause of our previously observed toxicity of casein amino acids, although the lot of cystine used in this experiment was slightly less toxic for *B. abortus* 1257 than that used in the original experiment.

Having previously observed that neither the Koser *et al.* (1941) nor the Mc-Cullough *et al.* (1947) amino acid medium would support growth of small inocula of *B. abortus* 1257, we decided to determine whether or not the high cystine content (150 μg per ml in the former and 192 μg per ml in the latter) of these media was the cause of this failure. When the cystine content of these media was lowered to concentrations tolerated by *B. abortus* 1257, this organism gave excellent growth from small inocula in both media.

We next set up an experiment to determine the maximum cystine tolerance of 42 strains of *Brucella abortus*, 10 strains of *Brucella suis*, and 9 strains of *Brucella melitensis*. For this purpose we used 1 per cent tryptose broth as the base medium. A maximum cystine concentration of 512 μg per ml was attempted by dissolving the required amount of the amino acid in the minimum necessary HCl, adding this to the base medium, and immediately adjusting the pH to 7.0. No precipitate was observed, but we doubt that the preparation retained this concentration of dissolved cystine. Additional concentrations of cystine ranging from 256 to 2 μg per ml of base medium were included in the experiment. Each test culture was adjusted to a uniform density (approximately 1 billion cells per ml) by means of an Evelyn photoelectric turbidimeter. Each test preparation received 0.1 ml of a 10^{-3} dilution of the adjusted test culture. The cultures were incubated at 37 C for 5 days with or without increased CO_2 as required.

TABLE 5

Species differences in the brucellae and strain differences in the Brucella abortus species with respect to the inhibitory action of cystine

SPECIES	NUMBER OF STRAINS TESTED	MAXIMUM NONINHIBITORY LEVEL OF CYSTINE, μG PER ML					
		16	32	64	128	256	512*
		Number of strains growing					
B. suis	10	—	—	—	—	8	2
B. melitensis	9	—	—	3	—	6	—
B. abortus	42 9 strains, require CO_2	—	—	4	5	—	—
	7 strains, adapted	4	2	1	—	—	—
	26 strains:						
	13 parents, require CO_2	—	—	11	2	—	—
	13 mutants, adapted	1	2	9	1	—	—

* Put into solution in HCl, added to the medium, and the pH adjusted to 7.0 immediately. No precipitate noted, but it is doubtful if this amount remained in solution.

Table 5 summarizes the results of this experiment. All 10 strains of *B. suis* tested tolerated 256 μg per ml of cystine, and 2 of them grew in 512 μg per ml. This is an interesting contrast with the 42 strains of *B. abortus*, none of which tolerated 256 μg per ml of the cystine. This observation may prove to be a significant aid in the taxonomic differentiation of these two species, particularly since the acclimated strains of *B. abortus* seem to become less rather than more tolerant to the toxic effect of cystine. The 9 *B. melitensis* strains tested showed cystine tolerances ranging down to 64 and up to 256 μg per ml. Thus they seemed to occupy a position intermediate between *B. abortus* and *B. suis* in cystine tolerance.

An experiment was performed to determine whether cystine was brucellacidal or only brucellastatic. Flasks of McCullough and Dick medium were prepared containing 5 and 50 μg per ml of cystine. These and a control flask containing no

cystine were each inoculated with 0.1 ml of a 10^{-2} dilution of a 48-hour broth culture of *B. abortus* 1257 and incubated at 37 C. One-half-ml aliquots of the contents of these flasks were plated at intervals for 72 hours. The flasks containing the cystine showed progressive decrease in numbers of *B. abortus* colonies and were sterile in 24 hours, whereas the control flask soon reached and retained a status of confluent growth. Thus the cystine toxicity for *B. abortus*, like the previously described peptone toxicity, is definitely brucellacidal.

Comparative toxicity of cystine and cysteine for B. abortus 1257. In an experiment designed to test a number of compounds related to cystine for toxicity for *B. abortus* 1257, we encountered an interesting zone of inhibition when cysteine was tested. Growth of the organism occurred at concentrations of cysteine of $6\pm$ µg per ml and below, and 100 to 200 µg per ml and above. We were inclined to believe that this zone of inhibition was the consequence of the partial oxidation of the cysteine to cystine, which resulted in a three-zone effect. At low concentrations of added cysteine not enough cystine was produced to be toxic.

TABLE 6

The effect of varying both the cystine and the cysteine concentration on the growth of Brucella abortus 1257

CYSTINE CONCENTRATION, µG PER ML	CYSTEINE CONCENTRATION, µG PER ML									
	400	200	100	50	25	12.5	6.25	3.12	1.56	0
	Growth in 5 days									
100	+	+	+	−	−	−	−	−	−	−
3.12	+	+	+	−	−	−	−	−	−	−
1.56	+	+	+	−	−	−	−	−	−	+
0	+	+	+	−	−	−	−	+	+	+

At intermediate concentrations of added cysteine there was adequate oxidation to produce toxic concentrations of cystine and not enough cysteine left over to exert a reversing effect on the cystine toxicity. At higher concentrations, the unoxidized cysteine tended to reverse the toxicity of the oxidized toxic cystine.

An experiment was designed to test this hypothesis. Graded concentrations of both cystine and cysteine were added to tubes of McCullough and Dick medium and the tubes were inoculated with 0.1 ml of a 10^{-2} dilution of a 48-hour broth culture of *B. abortus* 1257 and incubated at 37 C for 5 days. Table 6 records the results of this experiment and confirms both the marked toxicity of cystine alone (3.12 µg per ml) and the zonal toxicity of cysteine alone. Also this experiment indicates that the toxicity of 100 µg per ml of added cystine is reversed by 100 or more µg per ml of added cysteine.

In a second effort to confirm our hypothesis relative to the zonal toxicity of cysteine for *B. abortus* 1257, we repeated the experiment recorded in table 6 except that we substituted the reducing compound sodium formaldehyde sulfoxylate for the cystine. Our reasoning for this substitution was that the sodium formaldehyde sulfoxylate in adequate concentration would suppress the oxida-

tion of the cysteine and thereby restrict or eliminate the zone of toxicity by re-stricting or eliminating the production of toxic concentrations of cystine. Table 7 records the results of this experiment, which confirm our reasoning both as to partial restriction and as to complete elimination of the zonal toxicity of cysteine.

TABLE 7

Neutralization of the zonal toxicity of cysteine by the addition of sodium formaldehyde sulfoxylate to the medium

CYSTEINE CONCENTRATION, μG PER ML	SODIUM FORMALDEHYDE SULFOXYLATE, μG PER ML			
	12.5	6.25	3.12	0
	Growth in 5 days			
100	+	+	+	+
50	+	−	−	−
25	+	+	−	−
12.5	+	+	−	−
6.25	+	+	+	−
3.12	+	+	+	+
0	+	+	+	+

DISCUSSION

With the demonstration that cystine toxicity for brucellae correlated the toxicity of certain lots of tryptose with regard to inoculum-size effects, neutralization by reducing agents, and brucellacidal activity, we had hoped that we would be able to state definitely that cystine is the toxic factor in these peptones. Many additional experiments have been performed to test this hypothesis. These include antibacterial spectra of cystine and toxic tryptose, tolerance adaptations to these substances, paper chromatography, and microbiological and chemical analyses of the toxic and nontoxic tryptose for cystine. Most of these experiments support the contention that cystine is or could be the toxic factor in toxic tryptose, but the microbiological and other analytic tests fail to support the cystine hypothesis. This failure may be more apparent than real because of the possibility of a deficiency of neutralizing agents in the toxic peptones. These possibilities are being investigated and the results will constitute a portion of another paper dealing with the nature of the antibrucella factor in toxic peptones.

The observation of cystine toxicity for certain strains of brucellae, as demonstrated in this report, has practical significance in the formulation of synthetic media for these organisms. However, since acclimated strains of *Brucella abortus* seem to tend to be less tolerant to cystine than CO_2-requiring strains, we are inclined to believe that some factor other than cystine content is involved in the failure of synthetic media to support the growth of freshly isolated strains of this organism. Another practical aspect of the observed cystine toxicity which needs further study is the possible taxonomic value in the differentiation of *Brucella suis* and *Brucella abortus*. Also the reversal of cystine (including oxidized

cysteine) toxicity for brucellae by sodium formaldehyde sulfoxylate and other reducing agents would seem to justify the incorporation of some such agent in media receiving small inocula of these organisms.

The theoretical implications of the zonal toxicity of cysteine for *Brucella abortus* are most intriguing. If a similar antibrucella toxicity-inducing, toxicity-reversing mechanism could be demonstrated *in vivo*, it might account for the exacerbations and recessions of activity of the organisms as observed in clinical brucellosis. This conceivably would involve quantitative variations in the activity of the oxidases and reductases within the tissues. Also variations in the normal resistance or susceptibility to infection might involve some such reversible antibacterial compounds.

SUMMARY

Tryptophan and cystine were found to be highly toxic to *Brucella abortus* 1257 in a medium containing no other amino acids. Two additional amino acids, methionine and phenylalanine, were slightly toxic to this organism, whereas none of the other 15 amino acids of the casein digest series proved toxic at concentrations well above those found in 1 to 2 per cent casein digests. Only the cystine toxicity for this organism showed a persistence in the presence of other amino acids, which might implicate it as the antibrucella factor of casein and other digest media.

Cystine toxicity for brucellae correlates in many respects the previously reported tryptose toxicity for these organisms. However, we are not yet sure whether the two are or are not identical.

Forty-two strains of *B. abortus* showed markedly less tolerance for cystine than did 10 strains of *Brucella suis*. Acclimated strains of *B. abortus* tended to be less tolerant than CO_2-requiring strains. Nine strains of *Brucella melitensis* were intermediate in their tolerance for cystine.

The cystine content of certain synthetic media was shown to be responsible for the failure to obtain growth of some strains of *B. abortus*.

Cysteine tended to give zonal inhibition of growth of *B. abortus* 1257. This zone was explained in terms of the balance between the tendency of cysteine to be oxidized to cystine and the toxicity-reversing effect of residual cysteine. Varying amounts of the reducing agent sodium formaldehyde sulfoxylate restricted and, with increasing amounts, eliminated the zonal toxicity of cysteine.

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NUCLEI AND LARGE BODIES OF LUMINOUS BACTERIA IN RELATION TO SALT CONCENTRATION, OSMOTIC PRESSURE, TEMPERATURE, AND URETHANE[1]

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A number of investigators in recent years (cf. Robinow, 1945) have convincingly demonstrated the general, consistent occurrence in bacteria of discrete, Feulgen-positive units of chromatin, which in their various aspects have been referred to as chromatinic bodies, chromosomes, nucleoids, or nuclei. They may be seen in unstained cells, also, and have been recently identified in electron micrographs (Hillier, Mudd, and Smith, 1949). Much remains to be learned, however, concerning the fine structure of these bodies, their variation in gross form under normal conditions as well as under the influence of environmental factors, and their genetic significance, which is still purely a matter of inference.

The present study with marine luminous bacteria was undertaken with particular reference to the influence of salt concentration and osmotic pressure. These organisms are unusually favorable for the purpose in view inasmuch as comprehensive physiological, physical, immunological, and electron microscopical studies have been made in connection with the same factors (Harvey, 1940; Johnson, Zworykin, and Warren, 1943; Warren, 1945; Johnson, 1947). Optimal growth, luminescence, and respiration occur in sea water or isotonic (3 per cent) NaCl media, but if the cells are placed in greatly diluted salt solution (less than 0.3 per cent NaCl), respiration, viability, and growth cease; specific antigenic and surface-active substances are liberated into the medium; surface properties are affected; and "ghosts" remain in place of the normal cells. In isotonic (0.73 molal, or roughly 25 per cent) sucrose such changes do not occur at once, showing that the drastic effects are primarily osmotic. In concentrated sea water the cells shrink and their physiological activity and surface properties are again affected.

The physiological effects of salt concentration, of temperatures above the normal optimum, and of drugs such as urethane are largely reversible within certain limits of concentration and temperatures. Reversible changes also occur, under certain conditions, in the appearance of the nuclei, as shown by the present investigation. In addition, the hitherto unreported occurrence of "large bodies" in young cultures of these bacteria has been found.

Although species of both rod-shaped (*Achromobacter fischeri*) and coccoid cells (*Photobacterium splendidum*)[2] were studied, most of the work was done with the former, which provided somewhat more favorable material. Both species were routinely maintained on nutrient agar containing 3 per cent NaCl, 1 per cent glycerol, and about 0.5 per cent CaCO₃. For the nuclear stains, two successive plates of brain-heart agar plus 3 per cent NaCl were always prepared. The first was inoculated with a suspension of cells from a 1-day-old slant of maintenance medium. With *A. fischeri* this plate was incubated for 4 hours at the optimum temperature of 28 C, and the young growth was used to inoculate the second plate. The latter was incubated from 1.5 to 6 hours, as described later, usually at 28 C. Cultures of the psychrophilic species, *Photobacterium phosphoreum*, were incubated at 15 C.

The staining methods of Robinow, involving osmic fixation of cells on the agar surface, hydrolysis of impression films in N HCl at 55 C, and staining in dilute, phosphate-buffered Giemsa blood stain, were followed throughout.

The effects of tonicity and salts were studied in two ways. For the first method, permitting gradual changes, square holes, about 1.5 inches on a side, were cut in a sheet of cork, ¼-inch thick. Several strands of thin chromel wire were stretched across the under surface of the cork at each hole. A series of small agar blocks from young plate cultures could thus be mounted with their lower surfaces in contact with a solution of the desired concentration of salt or other constituents, to allow diffusion of the solutes between the agar block and the other solution. A large volume of the latter in an enamel pan was always employed. When a block from a luminescing culture on 3 per cent NaCl agar was thus placed against distilled water at room temperature, the brightness of the light gradually dimmed over a period of about 45 minutes and disappeared completely in slightly more than 1 hour. Calculations based on the rate of diffusion of NaCl through an agar block 2 mm in thickness indicated that most of the salt is removed within about an hour.

The reversibility of the effects observed after dialyzing against a given solution was tested by placing some similarly treated, but unfixed, agar blocks back on 3 per cent NaCl solution and allowing at least 1 hour for diffusion. The cells were then fixed and stained for nuclei by the standard procedure.

A second method, for the sudden reduction of salt concentration, was adding 1 ml of a 3 per cent NaCl suspension of cells from a young plate culture to 19 ml of distilled water, thus giving a final salt content of about 0.15 per cent, which results in almost instantaneous cytolysis. A portion of the diluted suspension was then spread on the surface of 2 per cent plain, nonnutrient agar containing no added salt, and agar blocks were fixed in osmic acid vapor in the usual manner. For rapid increases in salt concentration, a weighed amount of salt was added to a suspension of cells in isotonic solution. A portion was then

similarly spread on nonnutrient agar of a corresponding salt content, followed by osmic fixation.

Photomicrographs were taken with monochromatic light, using a Wratten filter no. 77 X to isolate the 546 mμ line in the emission spectrum of an AH-4 high-pressure mercury vapor lamp. A 95 X achromatic objective and 12 X ocular were used throughout.

RESULTS

Normal structure. Typical nuclear stains of coccoid and of rod-shaped cells of *Photobacterium phosphoreum*[2] and *Achromobacter fischeri*, respectively, each from young cultures at optimum temperatures, are shown in figures 1 to 4. In the former species (figure 1) the nuclear material appears variously as a darkly staining central body, or in two or more units distributed in a manner suggestive of changes similar to those accompanying mitosis in higher organisms. States resembling the anaphase are especially prominent, but examples are also evident which in appearance resemble the other phases of mitosis, including metaphase as seen in a polar view.

In *A. fischeri*, the chromatin of these young cultures appears mostly in the form of small granules, resembling the condition in older (18-hour) cultures of ordinary rods. The granules range in size from about 0.6 microns down to the limits of resolution of the microscope. The average size of distinct, apparently separate particles is greater than that of very small chromosomes, measuring 0.1 by 0.18 microns, in certain flowering plants (Blackburn, 1932 1933). The dumbbell-, X-, Y-, and V-shaped bodies, characteristic of the nuclei in young rods of common species (Robinow, 1945) are not so readily apparent in these preparations, although they can be detected by careful study (cf. figures 2a, 3a, and 4a). Moreover, such forms become conspicuous under the influence of increased salt concentration as described presently. Cultures of 1.5 to 2 hours' incubation at 28 C have long, snake forms (figure 2), which in the course of 3 or 4 more hours of incubation are largely replaced by numerous short and smaller cells (figure 3). The younger cultures frequently contain long filaments apparently devoid of nuclear material and some filaments in which the chromatin occurs as a continuous strand rather than as small discrete units.

The strain of *A. fischeri* used in this study dissociates, on maintenance medium, as dark colonies that are otherwise indistinguishable, through ordinary means, from those that emit light. Dark strains obtained from such colonies were examined, by the same procedure used for the luminescent cultures, for possible differences in appearance of the nuclei, but no differences were detected. Preliminary experiments were undertaken, from a genetic point of view, to determine whether luminescence would reappear in mixed cultures of dark variants, but the results have not been conclusive.

Large bodies of A. fischeri. Direct microscopic observations of living cultures showed that although "large bodies" were not present immediately after inoculation of a new plate from a 4-hour culture, they soon appeared, sometimes in considerable abundance (figure 4). They were produced from apparently normal

Figure 1. Young cells of P. phosphoreum; × 2,400.
Figure 2. A. fischeri, 1¾-hour culture; × 2,400.
Figure 3. A. fischeri, 6-hour culture; × 2,400.
Figure 4. A. fischeri, 4-hour culture, showing large bodies; × 2,800.

cells by a terminal or lateral swelling, which gradually enlarged and underwent changes in shape, in the manner illustrated by the diagrams in figure 5, resembling a similar phenomenon in cultures of Proteus (Hutchinson and Stempen, 1949). In the later stages, small granules were apparent within these bodies or at the

surface, seemingly in part on the outside. Although we have not succeeded in determining the fate of such bodies, which generally become overgrown by the more rapidly developing small cells, the granulated appearance suggests that they may ultimately give rise to the more typical cells, as in *Bacteroides* (Dienes and Smith, 1944) and in *Proteus* (Hutchinson and Stempen, 1949).
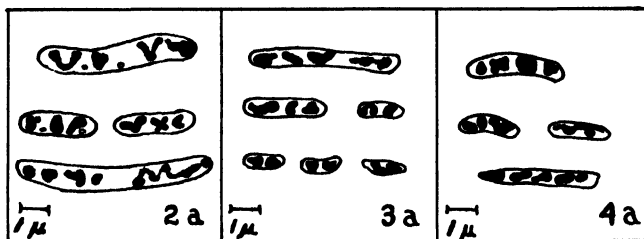
Figures 2a, 3a, and 4a. Diagrams, approximately to the scale indicated, of some of the cells in figures 2, 3, and 4, respectively.
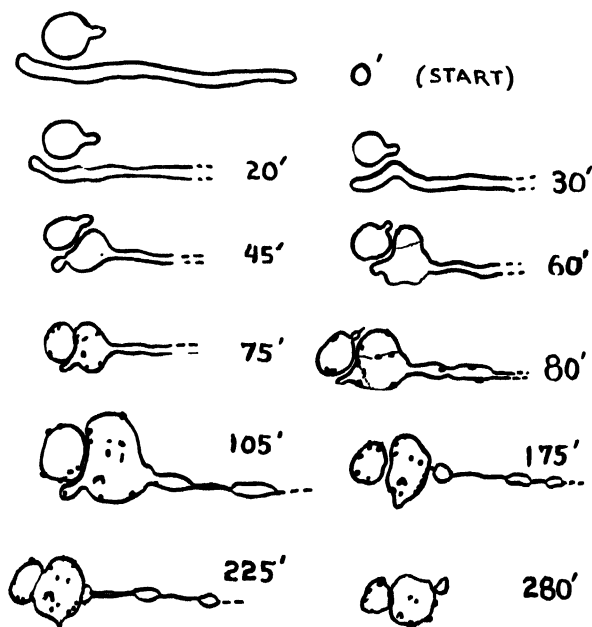
Figure 5. Diagram of formation of large bodies as observed in growing cultures of *A. fischeri.*

It was not unusual for two large bodies to form near each other on adjacent cells and to come into contact as they developed, as indicated in figure 5. There was no certain evidence that fusion ever occurred, although this possibility cannot be excluded on the basis of the observations that were made.

Among a large number of stained preparations exhibiting bodies with different appearances of the nuclear material, there were always some individuals that
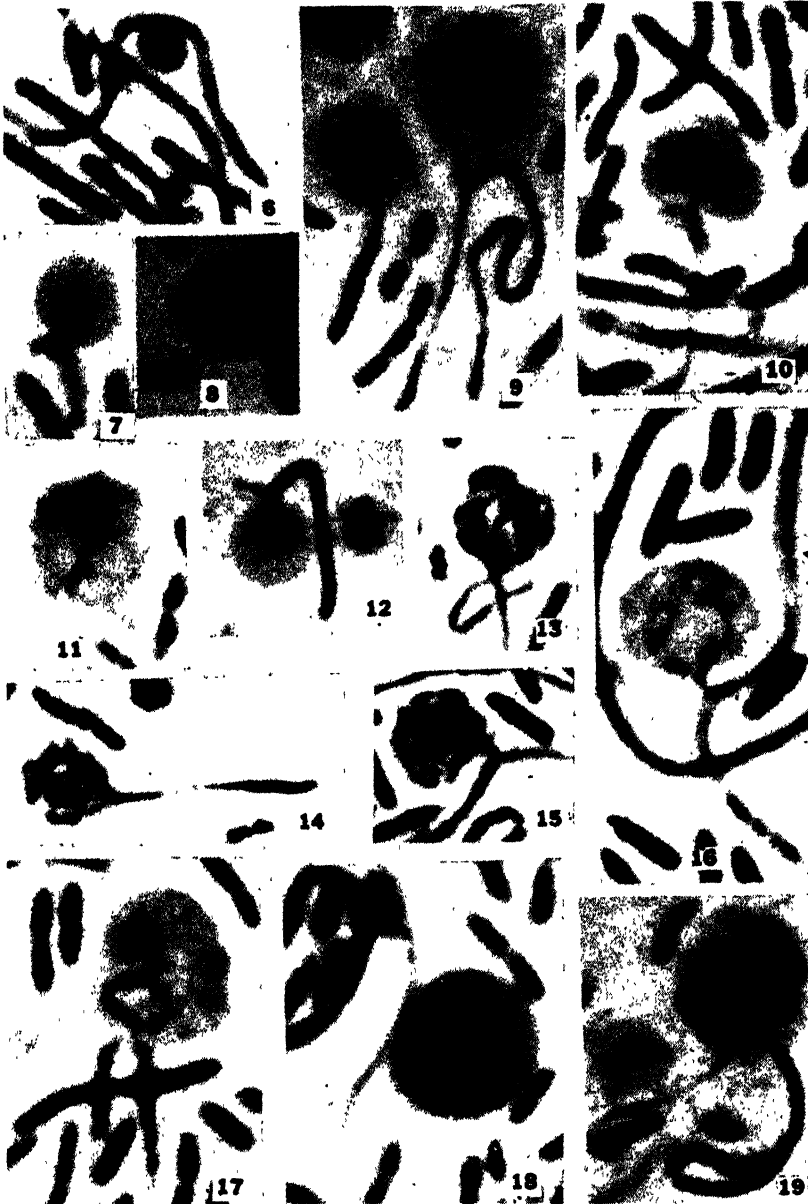
Figures 6 to 19. Representative large bodies in young cultures of *A. fischeri;* × 3,200. Figures 7, 11, and 16 to 18 from 5-hour cultures at 15 C; the others from 2- to 4-hour cultures at 28 C.

showed a general correspondence in their patterns. Representative examples are illustrated in figures 6 to 19.

Influence of hypotonicity. When agar blocks from a young culture are dialyzed against distilled water according to the method described, the nuclear material

gradually loses its stainability and discrete form as the solutes gradually diffuse out of the agar. An intermediate stage is illustrated in figure 20. The end result of prolonged dialysis is practically complete cytolysis (figures 22 to 24), in which the cells show only a vague or sometimes no clear indication of their former nuclear bodies. Moreover, they generally appear larger, as if by collapse accompanying loss of contents, somewhat like cells acted upon by lysozyme (Welshimer and Robinow, 1949, figures 3 and 4). The rate and extent of these changes were apparently greatest in the youngest cells (1- to 2-hour cultures), but the sequence and general picture were always the same.

At an intermediate stage of reduced salt concentration (e.g., as represented in figure 20) recovery of essentially the normal nuclear appearance takes place if the agar block is placed for an equivalent period of time in contact with 3 per cent NaCl solution (figure 25; compare with figure 2). This remarkable reversibility has a parallel in physiological activity in that both the reduced respiratory rate and the decreased luminescence intensity that occur in sea water diluted to 18 per cent of its normal concentration, by the addition of distilled water, undergo a large measure of recovery when concentrated sea water is added to restore the previous salt concentration (Johnson and Harvey, 1938). It is clear, therefore, that neither the functional activity of the cell nor the basic structure of the nucleus is destroyed by such treatment.

The preparation illustrated in figure 25 showed a somewhat unusual abundance of "X-bodies," i.e., long cells with a single, heavily staining, elongated central mass of chromatin, clear at each end, resembling those that have been observed by Robinow in other species of bacteria. Moreover, there appear to be intermediate stages toward the formation of normal cells from X-bodies, or vice versa (figures 26 to 32).

No reversibility of nuclear changes occurs after complete cytolysis resulting from either gradual or sudden reduction of salt beyond a critical concentration.

Distinction between hypotonicity and dilution of salts. When placed against isotonic sucrose, a temporary decrease in osmotic pressure in an agar block occurs because of the difference in rates of diffusion of NaCl and of sucrose. According to calculations, the minimum is reached at about 35 minutes, with the reduction amounting to 49 per cent. After 1 hour the initial osmotic pressure is largely restored, but the nuclear material of cells in the isotonic solution of the nonelectrolyte assumes an appearance similar in certain respects to that characteristic of incomplete cytolysis (figure 21). This change is largely reversible, as shown by subsequently placing similar blocks against 3 per cent NaCl for 1 hour. Thus, although osmotic pressure is apparently a contributing factor, the presence of electrolytes is obviously important to the normal state of the nucleus. The mechanism of these reversible changes in the nucleus is not clear, particularly because the plasma membrane is generally assumed to be practically impermeable to salt. The irreversible changes of complete cytolysis evidently result from the rupture of the cell, with irreparable disorganization of its structure, when the osmotic pressure gradient across the cell surface exceeds the mechanical strength of the wall.

Influence of concentrated salt solution. A gradual increase in salt concentration

Figure 20. Incomplete cytolysis of *A. fischeri* in dilute NaCl; × 3,300.

Figure 21. *A. fischeri* in isotonic sucrose; × 3,100.

Figures 22 and 23. Late cytolysis of *A. fischeri* in dilute NaCl; × 3,400.

Figure 24. *A. fischeri* from 6-hour culture, after removal of most of the NaCl, leaving no distinct nuclear structure; × 3,400.

to 20 per cent NaCl causes the chromatin to stain more intensively and apparently to coalesce into fewer but larger units with the dumbbell, V, and other shapes typical of those in young cultures of common species of rods (figures

Figure 25. "Reversed cytolysis" of *A. fischeri;* × 2,700.

Figures 26 to 32. Possible stages in the formation of an "X-body" from normal cells, or vice versa; × 2,700.

Figure 33. Effects of gradually increased NaCl to 20 per cent on nuclear structure of *A. fischeri*, 2.5-hour culture; × 3,400

Figures 34 and 35. Similar to figure 33, but with cells from a 4-hour culture, and counterstaining with fuchsin; × 3,400.

33 to 35). According to Robinow, older cultures (10 hours at 37 C) of the latter have more dispersed chromatin, which appears to coalesce when the cells are transferred to fresh medium, thus resembling the change in young cultures of *A. fischeri* under the influence of concentrated NaCl. The salt effect is reversible, but there was some indication that, after long contact with 20 per cent NaCl,

Figure 36. A. fischeri, after incubating for 80 minutes at 37 C.

Figures 37 and 38. Effects of sudden increase in NaCl to 20 per cent on nuclear structure of *A. fischeri;* × 3,100. The large body in figure 37 has an appearance suggestive of fusion or fission.

Figures 39 and 40. Cells from a 4-hour culture of *A. fischeri* after exposure to 0.5 M urethane for 35 minutes (figure 40) and for 63 minutes (figure 39), counterstained with fuchsin; × 3,100.

when the agar block was subsequently placed against 3 per cent NaCl, the cells underwent swelling, followed by changes resembling those that occur in a hypotonic medium.

Sudden increase in salt concentration, by direct addition to a suspension of cells, caused the chromatin to form an apparently continuous, wavy strand extending the length of the cell (figures 37 and 38). This change could not be reversed by the gradual removal of the salt.

Influence of above-optimal temperatures. At temperatures higher than 30 C, *A. fischeri* does not produce appreciable growth. When incubated for only 80 minutes at 37 C, the normal nuclear pattern of young cells appears disrupted, and the chromatin assumes the form of either a continuous or a slightly beaded thread (figure 36) somewhat like that resulting from a sudden increase in salt concentration. During subsequent incubation of such plates at room temperature, however, normal growth and luminescence took place, showing that not all the cells were killed by the fairly short exposure to 37 C. After incubation for several hours at this temperature, however, the nuclear structure varied from practically complete disintegration, only a few granules remaining, to strings of small, chromatinic spheres.

Influence of urethane. Depending upon concentration and temperature, urethane and related drugs catalyze a reversible and an irreversible denaturation of certain proteins both in living cells and in extracted systems. In effect, urethane lowers the temperature for thermal denaturation. In a concentration of 0.1 M, which causes a reversible inhibition of luminescence, there was no apparent change in the nuclei. At 0.2 M, which causes a greater inhibition of luminescence and a slow destruction of the system at room temperature, some pycnosis of the nuclear material could be detected. Higher concentrations of 0.4 to 0.5 M urethane, leading to irreversible physiological effects and preventing growth, also caused irreversible changes in the nuclei. The normally granular chromatinic structure was replaced even within a short period of time by a fairly dense, more or less continuous strand, or a line of small, apparently individual spheres (figures 39 and 40). These concentrations are of the same magnitude as those which Burt (1945) found prevented mitosis, caused pycnosis, and tended to revert the actively dividing nucleus of *Colpoda steinii* to the resting stage.

Significance of the effects of salt concentration, heat, and urethane. The changes in the nuclear material of *A. fischeri*, under the influence of the factors considered, are summarized schematically in figure 41. The double arrows indicate changes that are apparently easily reversible, and the single arrows those that are not. It is reasonable to believe that the latter type involves a denaturation of nucleoproteins by above-optimum temperatures, 0.5 M urethane at room temperature, or the sudden increase in NaCl concentration to 20 per cent.

In connection with this interpretation, the observations of Klieneberger-Nobel (1945) concerning various anaerobic species are of particular interest. Within a few minutes after exposure of the cells to air, the nuclear material begins to fuse and to take on an appearance resembling that in luminous bacteria after treatment with heat, urethane, or sudden increase in salt concentration (cf. her figures

6, 8, 10, 11, and 12). The well-known inimical effects of oxygen on obligate ana-erobes makes it reasonable to conclude that the mechanism of the nuclear changes in both cases involves protein denaturation.

The irreversible effects of greatly diluted salt solution, on the basis of various lines of evidence already referred to, involve the osmotic rupture of the cell, with loss of some of its contents and general disorganization of its structure.

The reversible changes under the influence of salt concentration and electro-lytes show that the nucleus of a given bacterial cell has a remarkable capacity to
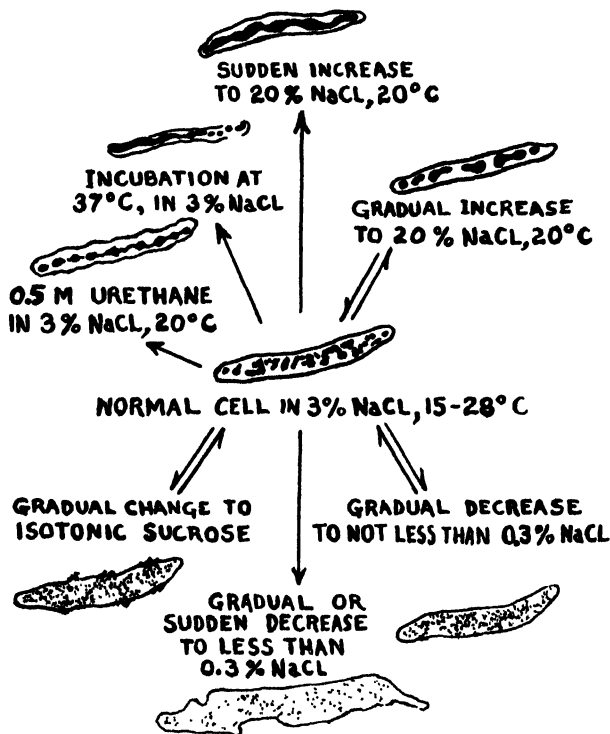
Figure 41. Diagrammatic representation of the changes in the appearance of the chroma-tin of A. fischeri under the various influences discussed in the text.

assume diverse appearances, varying from typical, densely staining Robinow bodies to seemingly small, dispersed, and lightly staining granules, without de-struction of its basic organization. The differences in appearances are, however, no more nor less remarkable than those accompanying normal mitosis and differentiation in cells of higher organisms. Thus, some of the variations in nu-clear structure that have been observed in other types of bacteria and within a single strain under different conditions, such as age of culture, are not surprising. They perhaps reflect merely the processes of normal cell division in bacteria and the influence of chemical differences in the internal cellular environment associ-ated with different stages of division, physiological states, or conditions of the medium. Although a complete definition of the bacterial nucleus must await the

accumulation of further evidence concerning its structure, organization, and function, it would obviously be a mistake to apply the term "nucleus" to any particular one of the diverse forms into which it reversibly enters.

Although the exact nature of the chromatin granules and their relation to individual nuclei in bacteria remain to be established, the influence of electrolytes, i.e., salt vs. sucrose, has a parallel in the influence of the same factors on the interphase nucleus of both plants and animals. Ris and Mirsky (1949) have shown that in nonelectrolyte solutions (sucrose, glycerol) the isolated nuclei stain lightly and homogeneously by the Feulgen method or by methyl green, specific for desoxyribonucleic acid (DNA). In physiological salt solution the chromatin structure becomes visible and the staining is more intense and confined to the visible structure. The effects are reversible on alternately placing the nuclei in sucrose and physiological salt solutions. Moreover, these changes are fully accounted for by changes in the state of the chromosomes, through a reversible extension and condensation of the DNA component. The behavior of animal and plant cell nuclei in strong salt solution is more complex, and is apparently not directly comparable to that of the nuclear material in the halophilic, marine luminous bacteria, but the similarities in the effects of electrolytes concern such a fundamental property of chromosomal material that it can scarcely be without significance. On this as well as other grounds, it seems very probable that there exists in bacterial cells a true homologue to the nuclei in plant and animal cells generally.

SUMMARY

The nuclei in marine luminous bacteria, *Photobacterium phosphoreum* (coccoid cells) and *Achromobacter fischeri* (rods), were studied by the staining methods of Robinow. In young cells of the former species on isotonic 3 per cent NaCl, brain-heart agar, the chromatin is distributed in a manner resembling various stages of mitotic cell division. In the latter species, young cultures consist mostly of long rods containing numerous small units of chromatin, but on the average larger than the smallest known chromosomes of higher organisms, varying in shape from spheroid to dumbbell, V, Y, and other shapes.

Large bodies occur abundantly in young cultures of *A. fischeri* on 3 per cent NaCl, brain-heart agar at optimum temperature. They form by lateral or terminal outpocketings from normal cells, but their ultimate fate was not determined. In stained preparations, the chromatin in large bodies has various appearances, but among numerous specimens some generally similar patterns are evident.

Dialysis of the salt from thin agar blocks of *A. fischeri* leads to cytolysis of the

cells, in the final stages accompanied by complete disintegration of the nuclear structure and rupture of the cells. Moderate reduction of the salt concentration, causing incomplete cytolysis, leads to a decrease in stainability of the chromatin, which also appears more finely dispersed, but this change can be reversed by dialyzing again against isotonic (3 per cent) NaCl. Dialysis against isotonic (0.73) molal sucrose causes reversible changes in appearance of the nucleus resembling those of incomplete cytolysis.

With *A. fischeri*, gradual increase in salt concentration of the medium to 20 per cent NaCl, by diffusion into thin agar blocks, causes the chromatin apparently to coalesce or aggregate into larger units, similar in appearance to the bodies found in young rods of ordinary bacterial species. This change is reversible on dialysis again against isotonic NaCl. Sudden increase in salt concentration, by the addition of an amount to make it 20 per cent in a suspension of cells, causes an irreversible change, the chromatin assuming the form of an apparently continuous thread extending throughout the length of the cell.

On brief incubation of *A. fischeri* at 37 C, about 10 degrees above the normal optimum, the chromatin tends to coalesce in the form of long, apparently continuous or sometimes beaded threads. At a much lower temperature, about 20 C, 0.5 M urethane causes an irreversible pycnosis of the chromatin into threads of spheroids resembling those that form under the influence of heat alone.

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LACTOBACILLI IN THE INTESTINAL TRACT OF THE CHICKEN[1]

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It has been shown that lactobacilli are numerically the most important group of bacteria in the intestinal tract of the chicken (Johansson *et al.*, 1948; Shapiro and Sarles, 1949). Isolation and partial characterization of 125 cultures from this source have revealed that these cultures of lactobacilli differ considerably from the intestinal lactobacilli found in other animals (Rettger *et al.*, 1935; King and Rettger, 1942; Porter and Rettger, 1940).

EXPERIMENTAL METHODS AND RESULTS

Isolation. Cultures of lactobacilli were isolated from carrot liver (CL) agar shake tubes (Garey *et al.*, 1941) inoculated with various dilutions of intestinal contents in work reported by Shapiro and Sarles (1949). Considerable difficulty was experienced in isolating the cultures. At first, colonies from the CL agar shake tubes were picked into CL broth before being plated for purification. However, the cultures grew very poorly in this broth, incubated aerobically or anaerobically (both an oat jar and a 100 per cent hydrogen atmosphere were used), and were soon lost through overgrowth by enterococci. The isolation procedure that proved most successful involved making stab cultures by picking colonies from the original CL shake cultures into fresh CL agar. The agar in the original shake tubes was aseptically blown into sterile petri dishes; the colonies were picked and stab-inoculated into fresh CL agar. These cultures were then used as a source of inoculum for the purification procedures. All "stab" cultures that showed the presence of gram-positive rods were streaked on CL agar for purification. Streak plates were incubated anaerobically in either an oat jar or in a hydrogen atmosphere (modified McIntosh-Fildes jar) at 37 C for 48 hours. Well-isolated colonies were transferred from the plates to CL agar stabs and maintained as stocks. Transfers of the stab cultures had to be made at 2- to 3-week intervals to keep the cultures viable.

An alternate procedure used to isolate some cultures of lactobacilli involved the inoculation of suitable dilutions of intestinal contents into plates of CL agar adjusted to pH 4.5. The plates were incubated anaerobically for 2 days, and isolated colonies were then transferred to CL agar (pH 7).

All cultures isolated were purified by plating and picking single colonies at least twice.

Occurrence. Isolations from colonies in CL shake tubes prepared from the

contents of various levels of the intestinal tract were made to determine the
distribution of lactobacilli in the intestinal tract. It was found that 25 to 50 per
cent of the colonies in CL agar shake tubes inoculated with colon contents were
lactobacilli, whereas in the CL agar shake tubes inoculated with cecal contents,
70 to 100 per cent of the colonies were lactobacilli. Colonies in the CL shake tubes
inoculated with contents of the ileum, duodenum, and gizzard were found to be
lactobacilli in almost all cases. The numbers of lactobacilli were highest in the
cecal pouches and decreased progressively in the colon, ileum, duodenum, and
gizzard (Shapiro and Sarles, 1949). Of 125 cultures selected for detailed study,
115 were isolated from cecal pouch contents. Of these, 68 cultures were obtained
from CL agar plates at pH 4.5, and 57 were obtained from CL agar shake tubes
at pH 7.0.

Morphology. The organisms are gram-positive, nonsporeforming rods, be-
coming gram-negative after 36 to 48 hours' incubation. The cells are short (2
to 4 μ) and thin (0.5 to 0.8 μ), occurring singly, in pairs, and in short chains,
and are commonly aggregated in masses resembling palisades. Marked chaining
does not occur nor does the cell shape change appreciably in the old, more acidic
cultures. Granulation may be detected in old cultures (48 to 72 hours) stained
with methylene blue.

Cultural characteristics. The colonies grow sparsely in petri dish cultures with
CL agar medium when incubated in an oat jar or in an atmosphere of 100 per
cent hydrogen. No growth is visible if the plates are incubated aerobically.
The colonies on CL agar plates are of two general types: one is light gray, trans-
lucent, small (1 to 2 mm in diameter), circular, flat, rough, with a depressed
center and very filamentous edges; the other is white, opaque, larger (3 to 4
mm in diameter), circular, raised, smooth, with entire edges. After 4 to 5 months'
incubation in CL agar, some of the cultures showing the rough colony type
changed over to the smooth type. Such a change did not cause alteration in other
characteristics of the organisms.

In CL agar stabs, all cultures grow only beneath the surface of the agar along .
the line of inoculation. Most of the cultures grow equally well throughout the
stab, but a few do not grow in the half-inch nearest the surface of the agar.
Agar stab growth may be said to be relatively profuse, uniform, and papillate
for most isolates. Growth in CL broth produces transient cloudiness with abun-
dant granular sediment, oftentimes deposited down the side of the tube.

Physiological characteristics. (a) Relation to free oxygen: The organisms are
all microaerophilic. Surface growth on CL agar does not occur on plates or
slants exposed to the atmosphere. Most of the cultures grow fairly well in plain
CL broth, but growth is enhanced by the addition of 0.1 per cent agar to the
medium. Incubation in an oat jar or in an atmosphere of 100 per cent hydrogen
also enhances growth. The addition of 5 mg ascorbic acid, 0.1 per cent sodium
thioglycolate, or 0.5 per cent glucose per 8 ml CL medium does not enhance
growth. All cultures are catalase-negative.

(b) Temperature relationships: The temperature relationships of the isolates
were determined in CL broth containing 0.15 per cent agar and adjusted to pH

7.0. Incubation was for 28 days at 18 C and 25 C, 14 days at 30 C, 10 days at 37 C and 45 C, and 7 days at 55 C. Growth was determined by visual inspection and by determination of the acidity of the cultures. Maximum growth was ascertained by determination of the final pH (electrometric) and the percentage of acid (expressed as lactic) at those temperatures for which turbidity differences could not be established.

The growth range and optimum temperature is approximately the same for all isolates. Although the optimum temperature, as indicated by acid production, seems to be slightly closer to 37 C than 45 C, the difference is not sufficient to warrant a statement of preference between the two. Growth is not initiated at 18 C, and only slowly at 25 C. The maximum temperature for initiating growth is between 45 C and 55 C; growth occurs at 45 C but not at 55 C.

(c) Growth curve: Forty-six cultures were inoculated into CL broth and incubated aerobically. Turbidity readings were taken with a Coleman junior spectrophotometer (660 mμ) at 2- to 3-hour intervals for 36 hours. Stock cultures of *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, and *Lactobacillus delbrueckii* were cultivated in the same manner. An appreciable increase in turbidity was not detected in most cultures up to 12 hours. From this time on, increases in turbidity were observed up to 26 to 28 hours. At this time, the turbidity of the cultures seemed to level off. The three stock cultures showed increases in turbidity beginning at 10 to 12 hours. It appears from this preliminary work that in the medium used, and when incubated aerobically, the CL broth cultures have a long lag phase (12 to 14 hours) and a log phase of from 12 to 24 hours. Rapid transfer of representative cultures in CL broth reduces the lag phase to approximately 8 hours. The size of the inoculum (within the limits used) apparently does not affect the growth curve for these organisms.

(d) Fermentations: (1) Litmus milk: Growth of these organisms in litmus milk indicates that they are not typically "dairy type" lactobacilli. Acid production in milk is slow, 48 to 72 hours before it becomes apparent by litmus color change, but on prolonged incubation curdling occurs. It is believed that the reaction produced in milk could be described as "slow acid curd" or ± curd, terms used to describe *Lactobacillus bifidus*. None of the cultures is a strong acid producer, since the highest acidity produced varied between 0.7 and 1.0 per cent expressed as lactic acid. (2) Carbohydrate utilization: Considerable difficulty was experienced in finding a suitable basal medium for studying the ability of the cultures to utilize various carbohydrates. Yeast extract glucose broth did not support good growth of these lactobacilli. Tryptone glucose yeast extract broth permitted better growth of the cultures, but the addition of beef extract greatly enhanced growth. Therefore, the basal medium adopted for all fermentation studies contained 1 per cent tryptone, 1 per cent yeast extract, 1 per cent beef extract, and 0.5 per cent of the carbohydrate. This medium was used as broth or with the addition of 0.1 per cent agar, and was adjusted to pH 6.8 to 7.0. All carbohydrates used were sterilized separately in 20 per cent aqueous solution by being autoclaved 10 minutes at 15 pounds steam pressure or by being filtered through a Seitz filter; they were added aseptically to the basal broth.

Carbohydrate utilization was determined by acid production indicated by an acid reaction with bromcresol purple after 7 days' incubation at 37 C. None of the cultures produce acid in the basal medium, although some may occasionally grow slightly. This basal medium gives typical results with "type cultures"[3] run concurrently with the isolates. The carbohydrates tested in this basal medium were glucose, fructose, galactose, maltose, sucrose, lactose, trehalose, and cellobiose. Salicin was the only glucoside employed. In addition, three alcohols were tested: sorbitol, mannitol, and dulcitol. The results are given in table 1.

On the basis of these fermentation tests the 125 isolates exhibit four patterns of utilization (7-day incubation):

Type I, constituting 72 per cent of the cultures, produces acid from glucose, fructose, galactose, maltose, sucrose, and lactose; no acid is produced from trehalose, cellobiose, and salicin.

TABLE 1

Carbohydrate utilization

CULTURE TYPE	PER CENT OF ISO-LATES	CARBOHYDRATE								
		Glu-cose	Fruc-tose	Galac-tose	Mal-tose	Sucrose	Lac-tose	Treha-lose	Cello-biose	Salicin
Type I	72	+	+	+	+	+	+	−	−	−
Type II	10	+	+	+	+	+	+	+	−	−
Type III	6	+	+	+	+	+	+	−	+	+
Type IV	10	−	+s	+s	+s	+	+	?	?	?
Type cultures:										
L. acidophilus {V203 {V204		+	+	+	+	+	+	+	+	+
L. bulgaricus V11		+	−	+	−	−	+	−	−	−
L. bulgaricus Gere A		+s	+	+	−	−	+	−	−	−

Explanation of symbols: +, utilized (acid to bromcresol purple at end of 7-day incubation period); +s, utilized slowly; −, not utilized; ?, inconclusive.

Type II, constituting 10 per cent of the cultures, produces acid from glucose, fructose, galactose, maltose, sucrose, lactose, and trehalose; no acid is produced from cellobiose or salicin.

Type III, constituting 6 per cent of the cultures, produces acid from glucose, fructose, galactose, maltose, sucrose, lactose, cellobiose, and salicin; no acid is produced from trehalose.

Type IV, constituting 10 per cent of the cultures, produces acid rapidly from sucrose and lactose, slowly from galactose, fructose, and maltose; acid is not produced from glucose. Trehalose, cellobiose, and salicin yield inconclusive results.

No utilization of the alcohols tested was observed with any of the cultures.

The remaining 2 per cent of the isolates either were lost in transfer or did not fit this scheme.

Type culture reactions: *L. acidophilus* V203 and V204—acid from glucose, fructose, galactose, maltose, sucrose, lactose, trehalose, cellobiose, and salicin.

L. bulgaricus VII—Acid from glucose, galactose, and lactose; no acid from fructose, maltose, sucrose, trehalose, cellobiose, and salicin.

L. bulgaricus Gere A—acid from galactose, fructose, lactose, and glucose (slowly); no acid from sucrose, maltose, trehalose, cellobiose, and salicin.

(3) Lactic acid production: Gas formation was not detected with any of the cultural procedures used, including CL agar shake tubes. The percentage of lactic acid produced from glucose was determined for representative cultures of each type and the "type cultures." Lactic acid was determined by the method of Barker and Summerson (1941), glucose by the method of Shaffer and Somogyi (1933). These determinations showed that between 75 and 90 per cent of the glucose utilized is converted to lactic acid. This indicates the cultures are homofermentative lactobacilli.

DISCUSSION

It is clear that these cultures of lactobacilli constitute a fairly homogeneous group. Approximately 72 per cent of the isolates appear to be identical. In this group are included cultures isolated from both CL agar at pH 7 and CL agar at pH 4.5. Although the use of the CL agar at pH 4.5 simplifies isolation by preventing the growth of other intestinal bacteria, it was not adopted as a routine procedure because it was believed that it might limit the species obtained. The results showed, however, that similar isolates were secured with CL agar at pH 7 and at pH 4.5, and, further, all cultures have been shown to be capable of initiating growth at pH 4.5. These cultures, particularly type I, differ considerably from *L. acidophilus*, the most commonly described intestinal lactobacillus. The cultures have shown stability in that the described characteristics of all isolates have remained virtually unchanged after one year of continuous transfer in CL agar stabs. The characteristics selected for study were those that would be of diagnostic value in identifying the cultures (Tittsler, 1948).

The cultures grouped in type IV are unable to utilize glucose that has been autoclaved separately, autoclaved in the basal medium, or sterilized by filtration. These isolates did not utilize glucose even after 2 weeks' incubation aerobically; some utilization may occur on prolonged anaerobic incubation. The inability of these cultures to utilize glucose is more marked than that of *L. bulgaricus* Gere A, described by Snell *et al.* (1948). This property of these cultures has remained stable for one year. It would be interesting to determine what conditions in the intestinal tract of the chicken favor the proliferation of such large numbers of non-glucose-fermenting lactobacilli. The apparently frequent occurrence of these cultures in the intestinal tract of the chicken is of interest in light of the statement by Snell *et al.* (1948):

The rapid rate of utilization of lactose or other disaccharides, as contrasted with the slow utilization of monosaccharides, may be much more common than the few recorded

instances would indicate. The common practices of following fermentation reactions qualitatively rather than quantitatively, and of recording action of an organism on a sugar as plus or minus after extended incubation periods, both tend to obscure this phenomenon, which is of considerable biochemical interest.

Further studies on the carbohydrate utilization of these strains are planned to determine whether or not these reactions are similar to those of other organisms that do not utilize glucose (Snell et al., 1948; Doudoroff et al., 1949).

More complete characterization of the lactobacilli will be necessary before their relationship to other members of the genus can be made clear. It is possible that they may prove to be strains of a new species.

SUMMARY

One hundred and twenty-five cultures of lactobacilli isolated from the intestinal tract of the chicken are described. The organisms are all catalase-negative, microaerophilic, homofermentative, short, gram-positive, nonsporeforming rods, whose optimum temperature is near 37 C, and which curdle milk slowly.

On the basis of carbohydrate utilization the isolates can be divided into four groups. The largest group (72 per cent of the cultures) produces acid from glucose, fructose, galactose, maltose, sucrose, and lactose, but does not utilize trehalose, cellobiose, or salicin. Ten per cent of the isolates do not utilize glucose even after prolonged incubation.

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FURTHER STUDIES ON THE BETHESDA GROUP OF PARACOLON BACTERIA[1]

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The Bethesda group of paracolon bacteria consists of microorganisms of uncertain pathogenicity frequently found in human feces. They constitute a rather uniform biochemical group. This group was studied by Stuart, Wheeler, Rustigian, and Zimmerman (1943) and classified by them as paracolon intermediate types. Barnes and Cherry (1946) reported 28 cultures of these organisms that were serologically related but were not subjected to complete antigenic analysis. The name "Bethesda group" was first applied by Edwards, West, and Bruner (1948) in a study of 32 cultures.

The pathogenicity of the group has not been established. The presence of these organisms in the feces of diarrheal cases that do not yield recognized pathogens continues to trouble the worker in enteric bacteriology and to cast a suspicion of pathogenicity on the group. The 28 cultures studied by Barnes and Cherry (1946) were isolated from one outbreak of mild diarrhea. The 32 cultures reported by Edwards, West, and Bruner (1948) were isolated from four distinct outbreaks of diarrhea.

Most of the 27 cultures reported here were isolated in routine examinations of feces of persons affected with diarrhea. Some were isolated from individuals who appeared normal. The group seems to be widely distributed and if the organisms are pathogenic it must be concluded that their pathogenicity is much lower than is the case with the *Salmonella* and *Shigella*.

In general the biochemical pattern established for the Bethesda group by Edwards, West, and Bruner (1948) is followed by the 27 additional cultures reported. Seven of the strains failed to ferment dulcitol, 10 fermented salicin in 6 to 30 days, none fermented sucrose in 30 days. Lactose was acidified by 26 cultures in 3 to 12 days, with 12 cultures producing gas. Mich 8 failed to produce acid after 30 days' incubation but resembled the others biochemically and produced the same characteristic bad odor. Although all the cultures produced acid in Jordan's tartrate, only five were able to utilize D-tartrate completely. Mesotartrate and L-tartrate were not completely utilized. All the cultures grew slowly on Simmon's citrate agar so that the medium became alkaline in 3 days, but three cultures were unable to utilize citrate completely. Mucate was utilized by all the cultures.

SEROLOGIC STUDIES

O serums were prepared for Na 1a, Na 4, Na 11, and Na 19 from broth cultures that had been heated at 121 C for 2.5 hours (Edwards, West, and Bruner, 1948)

and for Md 1, Md 3, and Mich 8 from broth cultures heated at 100 C for 3 hours. The serums used to study Mich 10 and Mich 11 were O and H combined serums prepared from formalinized broth cultures of these organisms.

The O antigens for the agglutination and agglutinin-absorption tests used throughout were broth cultures heated at 100 C for 1 hour and preserved with

TABLE 1

Agglutination reactions of somatic groups

ANTIGENS		SERUMS								
Cultures	O factors	Na 1a (1, 2)	Na 4 (1, 3)	Na 11 (1, 4, 5)	Md 1 (5, 6)	Md 3 (2, 7)	Mich 10 (7, 8, 9)	Mich 11 (7, 8, 10)	Na 19 (11)	Mich 8 (12)
Na 1a	(1, 2)	10,000	5,000	2,000	100	2,000	200	0	0	0
Na 4	(1, 3)	2,000	20,000	500	0	0	0	0	0	0
Na 11	(1, 4, 5)	200	100	20,000	200	0	0	0	0	0
Md 1	(5, 6)	0	0	2,000	1,000	0	0	0	0	0
Md 3	(2, 7)	500	0	0	0	5,000	2,000	5,000	0	0
Mich 10	(7, 8, 9)	0	0	0	0	5,000	10,000	10,000	0	100
Mich 11	(7, 8, 10)	0	0	0	0	2,000	5,000	10,000	0	200
Na 19	(11)	0	0	0	0	0	0	0	10,000	0
Mich 8	(12)	0	0	0	0	0	0	0	0	1,000

0 indicates no agglutination, 1:100. Figures in parenthesis indicate O factors.

TABLE 2

Paracolon diagnostic serums (O factors)

PARACOLON FACTORS	SERUM	SYMBOLS OF PARACOLONS	ABSORBING STRAINS AND PARACOLON COMPONENTS
1...	Na 11	1, 4, 5	Unabsorbed
2	Na 1a	1, 2	Na 4 (1, 3)
3	Na 4	1, 3	Na 1a (1, 2)
4	Na 11	1, 4, 5	Na 1a (1, 2) + Md 1 (5, 6)
5...	Na 11	1, 4, 5	Unabsorbed
6	Md 1	5, 6	Na 11 (1, 4, 5)
7	Md 3	2, 7	Na 1a (1, 2)
8...	Mich 11	7, 8, 10	Md 3 (2, 7)
9	Mich 10	7, 8, 9	Mich 11 (7, 8, 10)
10	Mich 11	7, 8, 10	Mich 10 (7, 8, 9)
11	Na 19	11	Unabsorbed
12	Mich 8	12	Unabsorbed

Three dots (. . .) indicate abbreviated formula.

0.3 per cent formalin. Tests were incubated at 50 C for 1 hour and read after standing overnight. Slide agglutination tests with alcohol-treated antigens gave comparable results but are not included here.

On the basis of agglutination and agglutinin-absorption tests nine O groups were recognized. The reactions of these groups are given in table 1. There are strong cross reactions among Na 1a, Na 4, and Na 11, between Na 11 and Md 1,

TABLE 3

Agglutination reactions of H antigenic types

Cultures	H factors	Na 1a (1)	Na 2c (2)	Na 23 (3)	Na 4 (4)	Na 22 (5)	Na 11 (5-6)	Md 3 (6, 7, 8)	Ala 20 (7, 9)	Mich 7 (8, 10)	Mich 1 (11)	Md 10 (12)	Mich 10 (13)	Conn 2780 (14)	Mich 11 (15)	Md 6 (16)	Mich 5 (17)	Mich 8 (18)	Ind 6 (19)	Na 921 (20)
Na 1a	(1)	10,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	500	0	0	0
Na 2c	(2)	0	10,000	1,000	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0
Na 23	(3)	0	400	10,000	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0
Na 4	(4)	0	0	0	10,000	500	1,000	0	0	0	0	0	0	0	0	0	0	0	0	0
Na 22	(5)	0	0	0	500	10,000	5,000	0	0	0	0	0	0	0	0	0	0	0	0	0
Na 11, ph. 2	(5)	0	0	0	10,000	10,000	10,000	0	0	0	0	0	0	0	0	0	0	0	0	0
Na 11, ph. 1	(6)	0	0	0	0	10,000	10,000	200	0	0	0	0	0	0	0	0	0	0	0	0
Md 3	(6, 7, 8)	0	0	0	200	500	200	10,000	5,000	5,000	500	200	0	0	0	0	0	0	0	100
Ala 20	(7, 9)	0	0	0	0	0	200	4,000	80,000	0	1,000	0	0	0	0	0	0	0	0	200
Mich 7	(8, 10)	0	0	0	0	0	500	5,000	0	20,000	500	0	0	0	0	0	0	0	0	500
Mich 1	(11)	0	0	0	0	0	0	0	0	0	10,000	5,000	500	0	0	0	0	0	0	0
Md 10	(12)	0	0	0	0	0	0	0	0	0	0	40,000	1,000	0	0	0	0	0	0	0
Mich 10	(13)	0	0	0	0	0	0	0	0	0	0	1,000	40,000	0	0	0	0	0	0	0
Conn 2780	(14)	0	0	0	0	0	0	0	0	0	0	0	0	40,000	1,000	0	0	0	0	0
Mich 11	(15)	0	0	0	0	0	0	0	0	0	0	0	0	2,000	10,000	0	0	0	0	0
Md 6	(16)	1,000	0	1,000	0	100	500	0	0	0	0	0	0	0	0	10,000	200	0	0	0
Mich 5	(17)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20,000	10,000	0	200
Mich 8	(18)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	200	10,000	0	0
Ind 6	(19)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	40,000	0
Na 921	(20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	40,000

0 indicates no agglutination, 1:100. Figures in parenthesis indicate H factors.

between Na 1a and Md 3, and among Md 3, Mich 10, and Mich 11. Except in the case of Md 3, which has no specific factor, absorption left good specific titers in each serum. The reactions among Na 1a, Na 4, and Na 11 are expressed by symbol 1, between Na 11 and Md 1 by symbol 5, between Na 1a and Md 3 by symbol 2, among Md 3, Mich 10, and Mich 11 by symbol 7, and between Mich 10 and Mich 11 by symbol 8. Preparation of the paracolon O factors by agglutinin absorption is indicated in table 2.

TABLE 4

Paracolon diagnostic serums (H factors)

PARACOLON FACTORS	SERUM	SYMBOLS OF PARACOLONS	ABSORBING STRAINS AND PARACOLON COMPONENTS
1	Na 1a	1	Mich 5 (17) + Na 1a (boiled)
2	Na 2c	2	Na 23 (3) + Na 2c (boiled)
3	Na 23	3	Na 2c (2) + Na 23 (boiled)
4	Na 4	4	Na 11 (5-6) + Na 4 (boiled)
5	Na 22	5	Na 22 (boiled)
6	Md 3	6, 7, 8	Ala 20 (7, 9) + Mich 7 (8, 10) + Md 3 (boiled)
7	Md 3	6, 7, 8	Na 11, ph. 1 (6) + Mich 7 (8, 10) + Md 3 (boiled)
8	Md 3	6, 7, 8	Na 11, ph. 1 (6) + Ala 20 (7, 9) + Md 3 (boiled)
9	Ala 20	7, 9	Md 3 (6, 7, 8) + Ala 20 (boiled)
10	Mich 7	8, 10	Md 3 (6, 7, 8) + Mich 7 (boiled)
11	Mich 1	11	Na 11, ph. 1 (6) + Mich 1 (boiled)
12	Md 10	12	Unabsorbed
13	Mich 10	13	Md 10 (12) + Mich 10 (boiled)
14	Conn 2780	14	Mich 11 (15) + Conn 2780 (boiled)
15	Mich 11	15	Conn 2780 (14) + Mich 11 (boiled)
16	Md 6	16	Unabsorbed
17	Mich 5	17	Na 1a (1) + Mich 5 (boiled)
18	Mich 8	18	Unabsorbed
19	Ind 6	19	Unabsorbed
20	Na 921	20	Unabsorbed

The H antiserums were prepared from formalinized broth cultures. Similar cultures were used as antigens in the H agglutination tests, which were read after incubation for 1 hour at 50 C. Nineteen H groups are established by serologic tests. These are set forth in table 3. Cross reactions occur between Na 1a and Mich 5, Na 2c and Na 23, Na 4 and Na 11, Mich 1 and Na 11, and Conn 2780 and Mich 11 which are not designated by common symbols. This omission has been in the interest of simplicity.

One-sided reactions also occurred, i.e., Mich 10 serum agglutinated Md 10 antigen but Mich 10 antigen was not agglutinated by Md 10 serum in a dilution of 1 to 100. These one-sided reactions that cannot be expressed by symbols appear to occur frequently in coliform cultures in both the O and H antigens. They have been reported by Kauffmann (1944), Wheeler (1944), and Edwards, West, and Bruner, (1947).

Na 11 (5–6) serum was prepared from the whole culture. This culture is diphasic and the two Na 11 antigens were cultures of selected colonies. Phase variation in this culture was discussed by Edwards, West, and Bruner (1948).

TABLE 5

Antigenic table

ANTIGENIC SYMBOLS		TYPE STRAINS	NUMBER OF CULTURES
O antigens	H antigens		
1, 2	1	Na 1a	17
	7, 9	Ala 20	1
1, 3	3	Na 23	2
	4	Na 4	6
	12	Md 10	1
	14	Md 8	2
	19	Ind 6	1
1, 4	2	Na 2c	6
	3	Na 12	4
	5	Na 22	1
	5–6*	Na 11	1
	6, (7), (8)	Md 4	1
	8, 10	Mich 7	1
	17	Mich 5	1
4, 5	6, (7), (8)	Md 2	2
	12	Md 1	1
2, 6	6, 7, 8	Md 3	1
	20	Na 921	1
6, 7, 8	13	Mich 10	1
6, 7, 9	15	Mich 11	1
10	3	Na 19	2
	11	Mich 1	1
	14	Conn 2780	1
	16	Md 6	1
11	18	Mich 8	1

Parentheses indicate that part of antigen is lacking.
An asterisk indicates diphasic culture.

The cross reaction between Na 11, phase 1, and Md 3 is expressed by symbol 6, between Md 3 and Ala 20 by symbol 7, and between Md 3 and Mich 7 by symbol 8. Preparation of the paracolon H factors by agglutinin absorption is indicated in table 4. Absorption left good specific titers in all the serums. Boiled homologous cultures were used in these absorptions to remove all O agglutinins.

ANTIGENIC TYPES

The antigenic types established are given in table 5. The nine O groups are divided into 25 serologic types, many of which are represented thus far by only one strain.

Md 2, which was assigned the H symbol 6 by Edwards, West, and Bruner (1948) to indicate its relationship to Na 11, phase 1 (6), is now assigned symbols 6, (7), (8). Further studies with these organisms have shown Md 2 to be closely but incompletely related to Md 3, that is, it removes all agglutinins from Md 3 (6, 7, 8) for Na 11 (6) and from Na 11 for Md 3, but it does not remove all agglutinins from Md 3 for Ala 20 (7, 9) and Mich 7 (8, 10); therefore only part of antigens 7 and 8 are present.

Type 1, 2: 1 is represented by 17 cultures. These are Na 1a, Na 1c, Na 3a, Na 3b, Na 3c, Na 24, Na 25, Na 26, Na 27, Na 28, Na 29, Na 31, Na 32, Na 33, Conn 2780, Md 11, and Mich 6. Type 1, 2: 7, 9 represents the type strain.

Type 1, 3: 3 is represented by 2 cultures: Na 23 and Md 7. Type 1, 3: 4 is represented by 6 cultures: Na 4, Na 5, Na 15, Na 16, Na 17, and Na 18. Type 1, 3: 12 represents the type strain. Type 1, 3: 14 is represented by 2 cultures: Md 8 and Md 9. Type 1, 3: 19 represents the type strain. Type 1, 4: 2 is represented by 6 cultures: Na 2a, Na 2c, Na 2d, Mich 12, Mich 17, and Mich 18. Type 1, 4: 3 is represented by 4 cultures: Na 2b, Na 12, Na 20, and Na 30. Types 1, 4: 5; 1, 4: 5–6; 1, 4: 6, (7), (8); 1, 4: 8, 10; and 1, 4: 17 represent the type strains.

Type 4, 5: 6, (7), (8) is represented by 2 cultures, Md 2 and Md B-3-46. Type 4, 5: 12 represents the type strain. Types 2, 6: 6, 7, 8; 2, 6: 20; 6, 7, 8: 13; and 6, 7, 9: 15 represent the type strains.

Type 10: 3 is represented by 2 cultures: Na 19 and St 1201. Types 10: 11; 10: 14; 10: 16; and 11: 18 represent the type strains.

SUMMARY

Further studies on the Bethesda group of paracolons, a biochemically uniform group, have demonstrated nine O groups that can be subdivided into 25 serologic types on the basis of their H antigens.

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DELAYED HYDROLYSIS OF BUTTERFAT BY CERTAIN LACTOBACILLI AND MICROCOCCI ISOLATED FROM CHEESE[1]

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Early work on the ripening of hard rennet cheese established that the enzymes concerned arise from one or more of the following sources: milk, rennet extract, and the bacterial flora of the cheese. Peterson, Johnson, and Price (1948a,b) have demonstrated, however, that enzymes of milk and rennet extract constitute only a small fraction of the total hydrolytic activity present in ripening Cheddar cheese.

Many studies on the role of bacteria in the ripening of Cheddar and other hard rennet cheeses have been made. In 1889 Adametz reported that the characteristic flavor did not develop in cheese that contained added thymol to retard bacterial growth. After further work revealed that the bacterial flora of normal hard rennet cheese consists predominantly of lactic-acid-producing bacteria, Adametz proposed the "lactic acid" theory of cheese ripening which recognized these bacteria as the principal cheese-ripening agent.

The majority of the bacterial studies on the ripening of Cheddar cheese since the work of Adametz support the "lactic acid" theory from the viewpoint of predominance of organisms, increase in the rate of flavor development, or increase in the rate of nonprotein nitrogen production. There have been, however, no reports based on adequate methods on the lipolytic activity of the bacterial flora of Cheddar cheese.

In previous work Peterson, Johnson, and Price (1948a) suggested that the lipolytic activity at pH 5 of Cheddar cheese may represent intracellular lipases of lactic acid bacteria liberated by bacterial autolysis. Peterson, Johnson, and Price (1949) further suggested that part of the free n-butyric and all of the free caproic, caprylic, and capric acids present in aged Cheddar cheese are the result of the action of these liberated bacterial lipases on the milk fat of the cheese.

In an attempt to determine the validity of the above hypotheses, studies on the intracellular lipolytic activities of pure strains of bacterial species present in large numbers in Cheddar cheese during ripening have been undertaken. It has been definitely established (Hucker, 1922a,b; Sherwood, 1939) that the organisms present in Cheddar cheese in sufficiently large numbers to function in the ripening process are included in the following groups: *Streptococcus, Lactobacillus*, and *Micrococcus*. The purpose of the present investigation is the study

of the intracellular lipases of a number of organisms isolated from normal Cheddar and brick cheese. These organisms fall in the *Lactobacillus* and *Micrococcus* groups. Bacterial isolates from brick cheese were also studied since the conditions of ripening and the bacterial flora of Cheddar and brick cheese are similar.

METHODS

Isolation and identification of organisms.[2] The *Lactobacillus* and *Micrococcus* organisms used in this investigation were isolated from cheese by the methods of Foster, Garey, and Frazier (1942) and Alford (1949), respectively. Group classification of all isolates and final identification of cultures shown to possess lipolytic activity were made by use of the scheme given in *Bergey's Manual* (Breed et al., 1948). All stock cultures of *Lactobacillus* isolates were carried in litmus milk and as stabs in a medium consisting of 0.25 per cent Difco yeast extract, 0.25 per cent glucose, 0.25 per cent Difco peptone, 1.5 per cent agar, and 5.0 ml each of mineral salt solutions A and B (Shull, Hutchings, and Peterson, 1942) per liter. Stock cultures of *Micrococcus* isolates were carried as stabs in a medium consisting of 1.0 per cent Difco tryptone and 1.5 per cent agar.

Preparation of constituents of media. Raw milk whey was prepared by rennet curd coagulation of whole milk adjusted to pH 5.75 with lactic acid. The turbid whey was adjusted to pH 7 and clarified by filtration through a coarse, diatomaceous filter aid (celite 545; Johns-Manville). The clear yellow whey was sterilized by passage through a Mandler bacteriological filter.

Purified butterfat was prepared as follows: An ether solution of fresh butterfat was washed with dilute sodium hydroxide, then with water. After removal of ether and water under reduced pressure below 50 C, the fat was stored in the refrigerator. Small portions (100 g) were sterilized just before use by autoclaving at 121 C for 2 hours.

Solid calcium carbonate was sterilized by being heated in an oven at 180 C for 6 hours.

Lipolytic activities of organisms. The lipolytic activities of isolated organisms were determined by measuring the extent of butterfat hydrolysis in whey butterfat cultures during a 60-day growth and autolysis period at 30 C. For each organism four identical sterile flasks were used. Each flask contained 100 ml of sterile whey and approximately 5 g of sterile calcium carbonate. To two of the flasks 3 g of sterile butterfat were added. One ml of inoculum from a 48-hour whey culture of the organism being studied was added to each of the four flasks. Several uninoculated control flasks, with and without butterfat, were set up for each run. In any single run only one preparation of raw milk whey and one of butterfat were used; thus the uninoculated flasks were controls for all the organisms included in that run. During the 60-day incubation period the cultures were shaken every second or third day to assure neutralization of acids formed or liberated. After growth was well under way (24 to 48 hours after inoculation),

the pH of the inoculated media for all organisms used in this investigation remained between 5 and 6 for the remainder of the 60-day period. The pH of normal Cheddar cheese during ripening lies between 5.1 and 5.3.

At 15 and 60 days, control and culture media, with and without butterfat, were analyzed for free volatile fatty acids by the following procedure: A suitable sample of each medium was adjusted to pH 2 with dilute sulfuric acid. The free fatty acids liberated were removed by extracting the acidified medium with
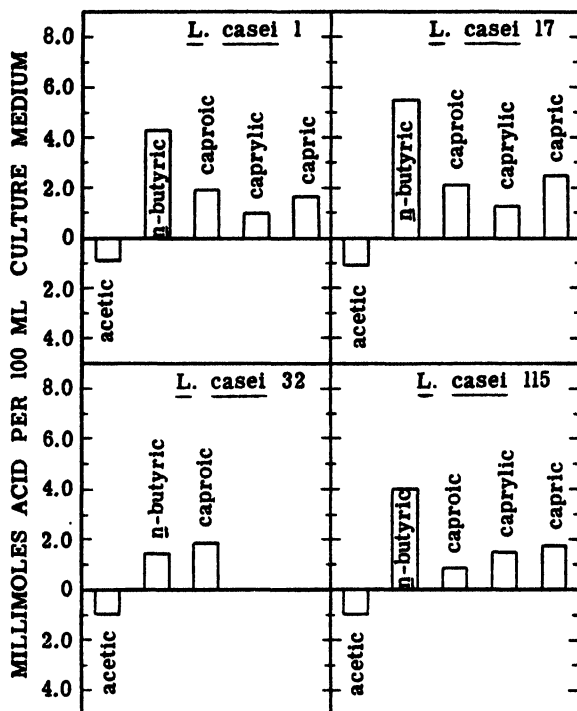
Figure 1. Butterfat hydrolysis between pH 5 and pH 6 by intracellular lipases of four *Lactobacillus casei* strains isolated from Cheddar cheese. The total block heights represent the levels of free fatty acids found in whey butterfat cultures incubated 60 days at 30 C. The block portions below the center zero line represent the fatty acid levels in similar cultures without added butterfat; hence the block heights above the center represent acids liberated from butterfat. In the case of acetic and *n*-butyric acids, the necessary corrections for small amounts of these compounds in the uninoculated media have been made.

ether. The acids were then removed from the ether extracts with dilute alkali and distilled from aqueous solution by a modification (Peterson and Johnson, 1948) of the magnesium sulfate procedure of Friedemann (1938). The distillate was titrated to the phenol red end point with 0.1 N sodium hydroxide. Although quantitative recovery of the total volatile free fatty acids is not possible by this procedure if free nonvolatile fatty acids are present (Smiley, Kosikowsky, and Dahlberg, 1946), the results obtained are sufficiently accurate for the purpose of this investigation. After suitable corrections for free volatile fatty acids pres-

ent in the uninoculated control flasks had been made, the amounts of free volatile fatty acid liberated by each organism in the presence and absence of butterfat were compared.

If any organism was found to have liberated more than 0.5 milliequivalents of volatile fatty acid per 100 ml of culture from butterfat, levels of individual fatty acids liberated were estimated by the method of Peterson and Johnson (1948). Analyses were made on all culture and control media of that organism. The identities of the fatty acids isolated during the partition chromatographic
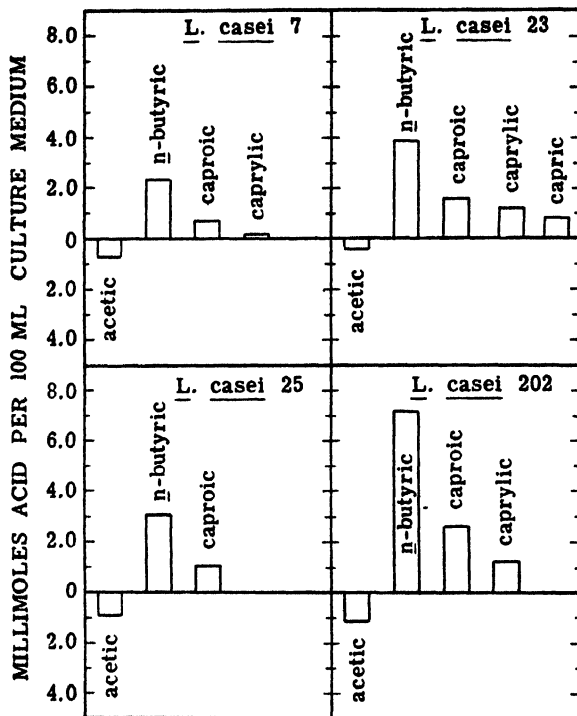
Figure 2. Butterfat hydrolysis between pH 5 and pH 6 by intracellular lipases of three *Lactobacillus casei* strains isolated from Cheddar cheese and one *L. casei* strain isolated from brick cheese (no. 202). See figure 1 for explanation.

analytical procedure were determined by their zone positions in column effluents. Fatty acids higher than capric acid, such as lauric, stearic, oleic, and so forth, were liberated only in trace amounts by the lipases of the organisms used in this investigation. Comparisons of butterfat hydrolysis at 15 and 60 days indicated whether lipolysis occurred during the normal life cycle of the organism or after autolysis.

LIPOLYTIC ACTIVITIES AND IDENTITIES OF ISOLATED CHEESE ORGANISMS

Sources and group classifications of isolated cheese organisms. Fifty-four *Lactobacillus* cultures from Cheddar and brick cheese and eight *Micrococcus* cultures

from raw milk Cheddar cheese were isolated. All *Lactobacillus* isolates are gram-positive rods with optimum temperatures between 30 C and 37 C. All strains produced lactic acid in litmus milk with subsequent curdling and litmus reduction. All *Micrococcus* isolates are gram-positive, catalase-positive cocci producing abundant growth on 1.0 per cent Difco tryptone agar.

Lipolytic activities of Lactobacillus isolates. After 60 days of growth and autolysis in the whey butterfat medium, only 12 of the 54 *Lactobacillus* isolates were found to have effected any appreciable butterfat hydrolysis. Seven of these 12 cultures were isolated from Cheddar cheese (numbers 1, 7, 17, 23, 25, 32, and 115)
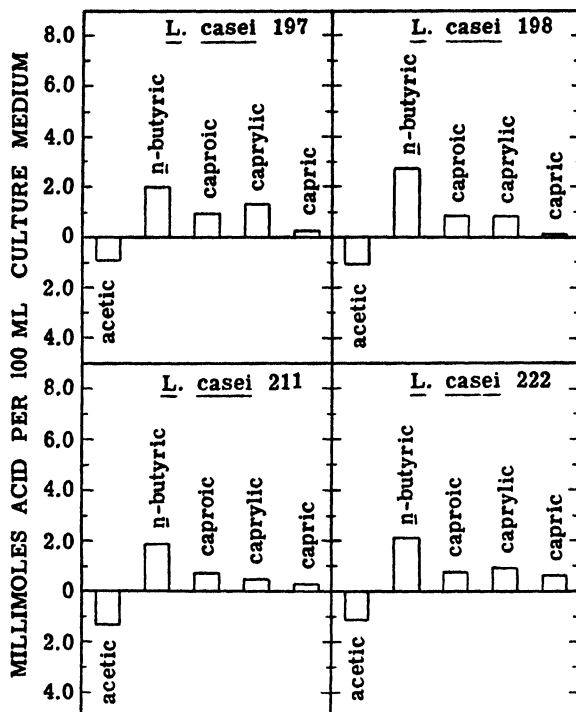
Figure 3. Butterfact hydrolysis between pH 5 and pH 6 by intracellular lipases of four *Lactobacillus casei* strains isolated from brick cheese. See figure 1 for explanation.

and the remaining 5 from brick cheese (numbers 197, 198, 202, 211, and 222). In no case was any lipolysis found to occur for any of these 12 isolates during the first 15 days of growth and autolysis at 30 C. Since these strains were all found to reach their growth peak in raw milk whey containing 0.5 molar acetate within 48 to 72 hours after inoculation, this demonstrates that these organisms are not appreciably lipolytic during their normal life cycles. Hence lipolysis occurring after 15 days and before 60 days was undoubtedly due to enzymes, active between pH 5 and pH 6, liberated by bacterial autolysis.

In figures 1, 2, and 3 the levels of individual fatty acids liberated from butterfat by the twelve *Lactobacillus* isolates having intracellular lipases active between

pH 5 and pH 6 are presented. A number of experiments were carried out, and
the data presented are the results of one representative experiment for each
isolate. As may be seen, there appears to be considerable lipolytic specificity
among the intracellular lipases of the isolates with regard to kinds and amounts
of fatty acids liberated.

Lipolytic activities of Micrococcus isolates. Although the eight *Micrococcus* iso-
lates were not found to be lipolytic during their normal life cycles, four of them
were found to possess intracellular lipases active between pH 5 and pH 6. In
figure 4 the levels of individual fatty acids liberated from butterfat by these four
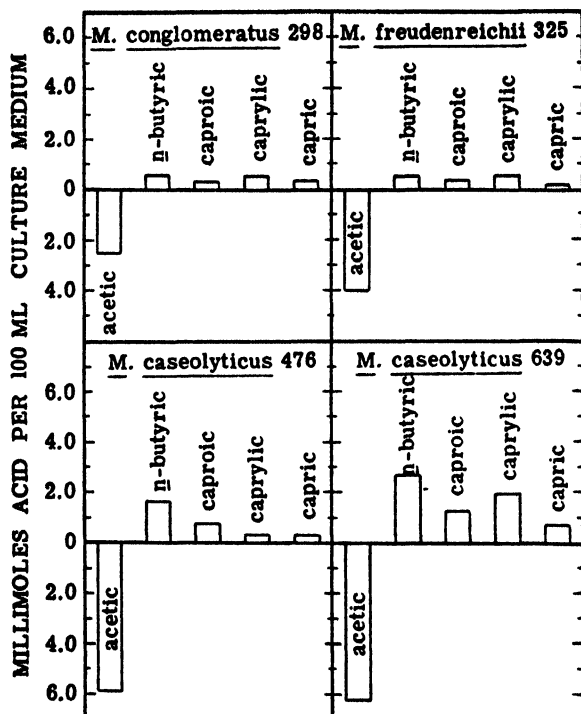
Figure 4. Butterfat hydrolysis between pH 5 and pH 6 by intracellular lipases of four
Micrococcus cultures isolated from raw milk Cheddar cheese. See figure 1 for explanation.

Micrococcus cultures are given. The data presented are the results of one of a
number of representative experiments for each isolate.

*Identities of Lactobacillus and Micrococcus isolates having intracellular lipo-
lytic activity.* All 12 *Lactobacillus* isolates having intracellular lipases active be-
tween pH 5 and pH 6 were identified as cultures of *L. casei.* These 12 cultures have
an optimum temperature between 30 C and 37 C, and curdle milk in 2 to 3 days
with reduction of litmus. All produce 1.1 to 1.5 per cent lactic acid in milk, are
nonmotile, are catalase-negative, do not liquify gelatin, and grow better ana-
erobically than aerobically. Sugar fermentations are identical in all cases with
those listed for *L. casei* in *Bergey's Manual* (Breed et al., 1948).

The 4 *Micrococcus* isolates having intracellular lipases active between pH 5 and pH 6 were identified as 1 culture of *M. conglomeratus*, 1 culture of *M. freudenreichii*, and 2 cultures of *M. caseolyticus*. Detailed identification studies on these *Micrococcus* cultures are presented by Alford (1949).

<center>DISCUSSION</center>

Although the 12 *Lactobacillus casei* cultures and 4 *Micrococcus* cultures shown to possess intracellular lipases active at the pH of normal Cheddar cheese were isolated from Cheddar and brick cheese, it would be very difficult to obtain proof that they function in cheese ripening. Although it is known that *L. casei* and *Micrococcus* organisms are present in Cheddar cheese in large numbers at various stages of ripening, a practical, differential bacterial count for organisms possessing intracellular lipolytic activity would be very difficult to devise. Indirect proof that bacterial organisms, such as the 16 studied in this investigation, function
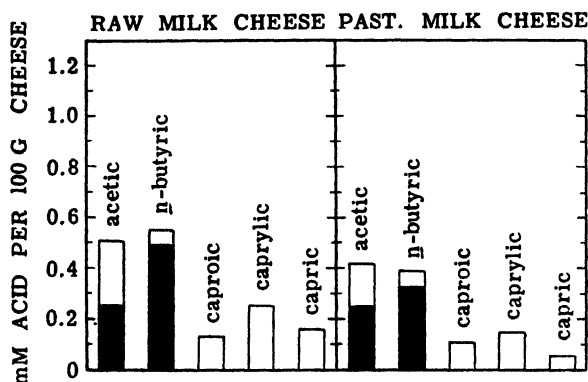
Figure 5. Free fatty acids present in Cheddar cheese. Total block heights, 360-day-old cheese; shaded block heights, 3-day-old cheese. Data from figure 1 (Peterson, Johnson, and Price, 1949).

in Cheddar cheese ripening might result from cheese-making studies in which milk cultures of these organisms are added individually and as mixtures to the cheese milk. Improved flavor development and rapid change of the individual free fatty acid picture to resemble that of aged Cheddar cheese (figure 5) would indicate activity of the added cultures. Alford (1949) found that the rate of flavor development in pasteurized milk Cheddar cheese to which milk cultures of *M. freudenreichii* 325 had been added was markedly increased. Fatty acid analyses, however, were not made.

<center>SUMMARY</center>

Twelve of 54 *Lactobacillus* cultures isolated from normal Cheddar and brick cheese and 4 of 8 *Micrococcus* cultures isolated from normal raw milk Cheddar cheese were found to possess intracellular lipases active between pH 5 and pH 6 and capable of considerable butterfat hydrolysis (3 to 12.8 milliequivalents of total fatty acid liberated per 100 ml of culture).

The 12 *Lactobacillus* isolates having intracellular lipolytic activity were identified as cultures of *L. casei*. The 4 *Micrococcus* isolates with similar lipolytic activity were identified as 1 culture of *M. conglomeratus*, 1 culture of *M. freudenreichii*, and 2 cultures of *M. caseolyticus*.

The intracellular lipases of 8 of the 12 *L. casei* cultures liberated *n*-butyric, caproic, caprylic, and capric acids from butterfat; the lipases of 2 of the other 4 *L. casei* cultures liberated *n*-butyric, caproic, and caprylic acids while those of the remaining 2 cultures released only *n*-butyric and caproic acids.

The intracellular lipases of all 4 *Micrococcus* cultures liberated *n*-butyric, caproic, caprylic, and capric acids from butterfat.

None of the *L. casei* or *Micrococcus* isolates found to have intracellular lipolytic activity showed lipolytic activity during their normal life cycles.

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NOTES

EFFECT OF VITAMIN B₁₂ ON THE PHOSPHORUS METABOLISM OF LACTOBACILLUS LEICHMANNII

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To study the incorporation of radioactive phosphorus by *Lactobacillus leichmannii* growing in media with and without vitamin B₁₂, the cells were grown either in a complete medium (broth plus liver extract) or in a test medium (Skeggs *et al.*: J. Biol. Chem., **176,** 1459, 1948) with a limiting quantity of B₁₂. After 48 hours' growth, the cells were harvested, washed, and resuspended in the test medium containing radioactive phosphorus. One one-hundredth μg per ml of B₁₂ was added to one-half of the cells, and the cultures were incubated at 37 C, either with aeration or in 100-ml graduates to give semianaerobic conditions. Twenty-ml samples were withdrawn at intervals for measurements of the growth (by optical density determination), pH, and radioactivity.

TABLE 1

Cells grown in complete medium

RADIOACTIVITY (COUNTS/SEC)	INCUBATION							
	Aerobic				Anaerobic			
	Time							
	1 hour		6 hours		1 hour		6 hours	
	Control	B₁₂	Control	B₁₂	Control	B₁₂	Control	B₁₂
	Optical density (Dλ₄₅₀)							
	0.066	0.066	0.155	0.194	0.066	0.066	0.170	0.256
Total..	96.4	86.1	392	464	75.0	74.1	630	852
Acid-soluble fraction	46.7	42.2	238	351	36.4	37.7	405	732
DNA fraction....	1.06	1.57	9.15	16.5	1.25	1.34	15.4	49.2

For the radioactivity measurements the cells were centrifuged, washed, and resuspended in sodium chloride. A sample was taken to measure the total radioactive phosphorus uptake, and the remainder was extracted with 5 per cent trichloroacetic acid (TCA) for 30 minutes at 5 C. Following this the precipitate (acid-insoluble fraction) was digested for 18 hours in 1 N KOH at 37 C, neutralized with HCl, and precipitated again with 2.5 per cent TCA to separate out the desoxyribonucleic acid (DNA). The radioactivity of all the fractions was measured

by sampling. As the sample of the original cells was very small, 0.1 ml of *Escherichia coli* cells was added before extraction to act as a carrier. The results of typical experiments are given in tables 1 and 2.

TABLE 2

Cells grown in test medium (anaerobic)

	TIME					
	0 hours		2 hours		8 hours	
RADIOACTIVITY (COUNTS/SEC)	Control	B₁₂	Control	B₁₂	Control	B₁₂
	Optical density D$_{\lambda 49}$					
	0.080	0.080	0.080	0.080	0.225	0.210
Total........................	157	163	995	1,050	4,510	7,750
Acid-insoluble fraction........	34	32.5	446	442	2,020	2,420
DNA fraction.................	2.6	1.7	14.8	18.1	67.9	260

Although the B_{12} causes an increase in the phosphorus uptake of all the fractions, the effect is particularly noticeable in the DNA fraction. In the experiment of table 2, for example, the growth was the same in both cultures, but the DNA fraction of the B_{12} sample shows four times the radioactivity of the control. These observations are in accord with the concept that B_{12} is involved in nucleic acid synthesis (Shive *et al.*: J. Am. Chem. Soc., **70**, 2614, 1948; Snell *et al.*: J. Biol. Chem., **175**, 473, 1948).

THE INFLUENCE OF VITAMIN B₁₂ ON THE GROWTH OF BACTERIOPHAGE T4r

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In the preceding note (Roberts *et al.*: J. Bact., **58**, 709, 1949) the influence of vitamin B_{12} on the phosphorus metabolism of *Lactobacillus leichmannii* was described, the most prominent effect being found in the desoxyribose nucleic acid (DNA) fraction. Since it is known (Cohen: Bact. Revs., **13**, 25, 1949) that DNA synthesis is important in the multiplication of the bacteriophage T4r, it seemed likely that B_{12} might also be involved in the growth of this virus. No requirement for B_{12} is normally observed as the host cell, *Escherichia coli*, strain B, is capable of synthesizing B_{12} from a synthetic medium, and the growing cells might be expected to have an adequate supply of this vitamin. Abelson (unpublished) has found, however, that the B_{12} content of the cells is markedly reduced in the resting phase. Also, it is known that the burst size of virus from resting cells is

small and variable, which might be an indication that B_{12} is a limiting factor in this particular case.

Bacteria were grown in synthetic medium containing ammonium chloride, phosphate buffer, magnesium sulfate, and glucose. The cells were centrifuged and washed free of the original medium at the desired phase of growth. Three conditions were used, namely, growing cells, resting cells that had grown to saturation, or cells that had stopped growth abruptly during the exponential phase because of a lack of glucose ("starved cells"). One-step growth curves were made by the usual technique (Cohen: *loc. cit.*; Anderson: Botan. Rev., **15,** 467,

TABLE 1

Effect of B_{12} on virus growth in starved cells stored 4 days in refrigerator before infection

CONCENTRATION OF B12 (µG/ML)	TIME AFTER INFECTION (MIN)									
	20	40	45	50	55	60	70	85	100	120
	Number of plaques									
0	2	7	7	6	8	4	9	18	21	20
8×10^{-6}	8	4	5	11	15	14	17	17	25	24
2.5×10^{-4}	10	5	10	11	29	35	23	41	36	30
7.5×10^{-3}	5	7	15	22	25	32	50	44	34	47
2.4×10^{-1}	2	8	12	23	54	43	55	70	53	45

TABLE 2

B_{12} effect with cells in various conditions

(Concentration of B_{12} 0.2 µg per/ml. Accuracy of burst size ± 10 per cent)

STATE OF CELLS	TIME OF BURST (MIN)		AVERAGE BURST SIZE	
	Control	B12	Control	B12
Growing cells..........	35	35	77	85
Resting cells...	45–60	45–60	9	15
Starved cells...	20–45	20–45	34	39
Starved cells stored 1 day in refrigerator.. ..	40–60	40–60	13.5	19
Starved cells stored 2 days............	55	42	4	10
Starved cells stored 4 days..	75	45	3.3	8

1949) using the synthetic medium with tryptophan added. A large excess of bacteria was used giving a bacteria to virus ratio of approximately 100. After a 5-minute absorption period (approximately 90 per cent absorption) the infected cells were diluted (1:25,000), and the final suspension was divided into two halves, B_{12} being added to one. Plates were made from these growth tubes at intervals. This technique avoided any dilution errors in comparing growth with and without B_{12}, but it should be noted that the B_{12} was not added until 5 minutes after infection.

We have found larger bursts as well as decreases in the burst times when B_{12} was added to the medium. Four different commercial vitamin B_{12} products (two

crystalline B_{12} and two concentrated liver extracts) used at a concentration of 0.1 μg of B_{12} per ml gave comparable results, the extracts showing slightly larger bursts than the crystalline preparations. Table 1 shows the results obtained using various concentrations of B_{12}, and the variation of B_{12} effects with the state of the cell is shown in table 2. Since all of the different products gave essentially the same result and the B_{12} was effective even at high dilutions, it seems most reasonable to attribute the effect on virus growth to B_{12} and not to some unspecified component of the solution.

It thus appears that B_{12} is one of the rate-limiting factors in virus synthesis of resting cells, the effect showing both in the burst time and in the average burst size. The cells of the host do not contain sufficient B_{12} to provide one molecule per virus particle. Neither is there any large increase in B_{12} synthesis during virus infection. Consequently, it appears that B_{12} is not utilized as a component of the virus. Because of its effect on the DNA formation of *L. leichmannii* and its relationship with thymine (Shive *et al.*: J. Am. Chem. Soc., **70**, 2614, 1948; Snell *et al.*: J. Biol. Chem., **175**, 473, 1948), it seems probable that the B_{12} effect on virus multiplication is through its role in thymine synthesis.

Two applications of these findings are possible. One is the use of virus growth as a biological assay for the B_{12} vitamin, as demonstrated in table 1. A second is the use of suitable B_{12}-inactivating or B_{12}-competing agents in virus-infected cells as means of chemotherapy.

We wish to thank Dr. Hugh Darby for his encouragement and interest in these experiments.

UNUSUAL STRAINS OF DIPHTHERIA BACILLI FROM THROAT CULTURES

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Aberrant strains of diphtheria bacilli have been encountered more frequently during the past five years than previously. Unusual strains have been reported in the *Annual Reports of the Division of Laboratories and Research*, 1944 to 1948. Frobisher and others (Proc. Soc. Exptl. Biol. Med., **58**, 330, 1945), and Jebb (J. Path. Bact., **60**, 403, 1948) have reported irregularities in strains of *Corynebacterium* from the nose and throat. A strain studied in this laboratory in 1948 was notable for its invasiveness in guinea pigs, and another, isolated in 1949, for its fermentative properties. The former was recovered from the heart's blood of 6 of 14 guinea pigs inoculated intracutaneously or subcutaneously. It was isolated from throat cultures from two boys, 15 and 13 years of age, students in the same school. One was said to have had "acute pharyngitis" and the other, "sore

throat." Diphtheria antitoxin was reported to have been given to the first but not the second. Morphologically typical diphtheria bacilli were found in several other throat cultures from each patient, but no attempt was made to isolate and study them. This strain, no. 4876, had the morphologic, cultural, and biochemical properties of the diphtheria bacillus.

The first series of tests in guinea pigs consisted of intracutaneous tests on two animals, one of which had received 1,000 units of diphtheria antitoxin. Each guinea pig received four injections (two of each culture) consisting of 0.1 ml of a broth suspension matching in turbidity pyrex standard no. 0.5. The unimmunized guinea pig died within 48 hours but was not autopsied. The guinea pig that had received antitoxin died 12 days after inoculation; diphtheria bacilli were recovered from the sites of inoculation and from the heart's blood. A second series of tests consisted of two intracutaneous tests on each of four guinea pigs, two of which had received 1,000 units of antitoxin. Both unimmunized guinea pigs died within 48 hours; no diphtheria bacilli were recovered from the sites of inoculation or from the heart's blood. The animals that had received antitoxin were protected. Eight guinea pigs were inoculated subcutaneously with 2 ml of 72-hour broth cultures. Two that had received 1,000 units of diphtheria antitoxin became emaciated and developed extensive edema and necrosis at the sites of inoculation. They were chloroformed and autopsied eight days after inoculation; diphtheria bacilli were recovered both from the sites of inoculation and the heart's blood. The six unimmunized guinea pigs died within 24 hours of inoculation; diphtheria bacilli were recovered from the heart's blood as well as from the sites of inoculation of three. The autopsy findings, especially in the animals that had not received antitoxin, were those of diphtheritic toxemia. The recovery of the microorganism from the blood stream suggests that the failure of antitoxin to protect against the standard inoculum was because of the invasiveness of the strain.

A virulent strain of diphtheria bacillus studied in 1949 (no. 4961) fermented glucose slowly but neither sucrose nor dextrin. It was isolated from a throat culture from a woman, 41 years of age, who had been ill for two days; the clinical diagnosis was tonsillitis or diphtheria. This strain is of particular interest because microorganisms morphologically typical of diphtheria bacilli and having these fermentative properties are frequently isolated from throat cultures, but previously all have been nonvirulent for guinea pigs.

Bacteriologists engaged in diagnostic work should thus bear in mind that antitoxin may not protect guinea pigs against inoculations with strains of diphtheria bacilli that are invasive, and that every microorganism having the morphology of the diphtheria bacillus, regardless of fermentive properties, should be regarded as virulent until proved otherwise.

ADAPTIVE ENZYMES INDUCED BY INSOLUBLE SUBSTRATES

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The chemical reaction by which a substrate induces a cell to form an adaptive enzyme is not known. Cells may produce many highly specific proteins in response to a specific stimulus. Enzyme production is only one special case; antibodies generally belong to this category (Rahn: Growth, **2**, 363, 1938).

It has recently been shown by Rothen (J. Biol. Chem., **168**, 75, 1947) that the reaction between antigen and antibody is due to an attractive force operating over a distance that is very short but still within the range of microscopical visibility. The formation of an adaptive enzyme in response to a specific substrate can be considered as a similar case. We can look at the enzyme as a mold or template made to fit a certain part of the substrate.

It is commonly assumed that enzymes are formed within the cell, and ordinarily the substrates can diffuse into the cell to serve there as templates. This leads to the question whether insoluble substrates, such as starch, or fat, or protein, can induce the formation of adaptive enzymes. Our experiments with the amylase of *Streptococcus bovis* seem to answer this question in the affirmative. Experiments with "soluble starch" showed that *Streptococcus bovis* produced no amylase when grown without any carbohydrate; a small amount was formed when grown with glucose and 15 times as much when grown with starch. The amount of amylase was measured by the length of time required by a mixture of 20 ml of cell-free culture (centrifuged) and 1 ml of a 2 per cent starch solution to hydrolyze all starch so that no blue color appeared with iodine on the spot plate. The amylase of *S. bovis* is quite sensitive to acidity; its optimal pH is at 6.55; good reaction is obtained between pH 6.35 and 7.0. Therefore, the reaction of the centrifugate was adjusted with phosphate buffer or $CaCO_3$ before testing for amylase.

A typical result is the following: *Streptococcus bovis* was grown in nutrient broth without or with glucose and starch as indicated in the table. The cultures were centrifuged, and the clear supernatant, after the addition of starch, was tested at short intervals on the spot plate to see how much time was required to hydrolyze all the starch.

Percentage of starch added........	0	0.2	0	0.1	0.05	0.15
Percentage of sugar added.........	0	0	0.2	0.1	0.15	0.05
Time for complete hydrolysis.....	> 3 days	8 hr	120 hr	8 hr	8–12 hr	8–12 hr

It is evident that absence of starch in the culture medium results in a very small amount of amylase, whereas the addition of only 0.05 per cent produced

plenty of it, and 0.2 per cent did not produce significantly more amylase than 0.05 per cent.

The pH of these experiments varied only between 6.2 and 7.0, and, in this range, the rate of amylase action is nearly constant, and nearly optimal.

Summary. Though a small amount of amylase is formed by *Streptococcus bovis* in the presence of glucose, the amylase production is increased about 15 times by the presence of starch. How a nondiffusible substrate can cause this stimulation is not clear. We may assume that enzymes are formed on the outside of the plasma membrane, or we may resort to a reaction similar to Rothen's observation of a distant action between antigen and antibody.

STAPHYLOCOCCAL HYALURONIDASE[1]

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Several investigators have been interested in the hyaluronic acid, hyaluronidase system in bacteria and its relation to invasion and virulence. Duran-Reynals (1933) correlated the invasiveness of staphylococci and streptococci with the yield of diffusing factor. Kendall and co-workers (1937) demonstrated hyaluronic acid in the culture media of three types of group A hemolytic streptococci in the mucoid phase, and Seastone (1939) isolated hyaluronic acid from group C hemolytic streptococci in the mucoid phase. Seastone (1943) also showed that 94 per cent of strains from moderate or severe streptococcal infections in man have been found to produce the mucoid polysaccharide in varying amounts. In a group of streptococci from normal throats only about 8 per cent produced hyaluronic acid, all of the producers falling into Lancefield's group A. McClean (1941) demonstrated that capsules and hyaluronidase do not coexist in the same group A or C strain of streptococcus.

McClean (1936) found that hyaluronidase is produced by organisms of the gas gangrene group, and McClean and Hale (1941) showed that the inclusion of potassium hyaluronate in the culture medium of *Clostridium perfringens* resulted in increased production of the enzyme. From this fact he postulated that the presence of hyaluronic acid *in vivo* increases enzyme production, setting up a vicious circle that promotes rapid extension of the infection.

Assay of hyaluronidase. The assay method used was that of Tolksdorf *et al.* (1949), which was kindly made available to us prior to publication. We introduced the following minor modifications:

(1) The potassium hyaluronate was dissolved in 0.1 M acetate-sodium-chloride buffer, pH 6.0, to give the recommended transmittance of 50 ± 5 per cent at a wave length of 600 mμ. A standard curve of turbidity development by hyaluronate and acidified protein showed a transmittance of 47 per cent with a concentration of 0.2 mg per ml. Dilutions of substrate ranging from 0.06 mg per ml to 0.2 mg per ml yielded absorbencies proportional to concentrations of hyaluronate. Therefore, the concentration of 0.2 mg hyaluronate per ml of buffer was used. As the solution ages, the turbidity obtained decreases slightly. The solution is kept in the cold and discarded after 3 weeks. Potassium hyaluronates supplied by the Schering Corporation and by the Wyeth Institute have been used.

(2) Horse serum was used in place of human plasma in the preparation of the acidified protein solution.

(3) The Coleman junior spectrophotometer was used in the measurement of turbidities.

A search of the literature for references concerning nonenzymic factors that might depolymerize hyaluronate shows that the culture media contain nothing likely to cause false results. Favilli (1940) found that an azoprotein prepared from diazobenzene sulfonic acid coupled with horse serum would reduce the viscosity of synovial fluid. The rate differs from that of hyaluronidase, and pH has very little effect on the reaction. Madinaveitia and Quibell (1941) found that ascorbic acid and certain diazo compounds could cause a fall in the viscosity of hyaluronic acid but that the reaction is independent of pH. Robertson et al. (1941) found that ascorbic acid in the presence of H_2O_2 brings about a degradation of synovial mucin; but this is not accompanied by the liberation of reducing sugar.

As a precaution a few "blanks" of sterile media were run for possible turbidity reduction. None showed any reduction.

Production of hyaluronidase. Tryptic digest (pH 7.6) was selected as the culture medium for the following reasons: (1) It has been established (Rogers, 1945) that optimal formation of hyaluronidase occurs only in well-buffered media. (2) Previous studies of staphylococcal coagulase production, made in this laboratory (Walker et al., 1947, 1948), have shown that this medium is highly satisfactory for the growth of the staphylococcus.

Four strains of *Staphylococcus aureus* were assayed quantitatively for hyaluronidase production: L—isolated in April 1946 from a mastoid infection; known to be hemolytic and to produce coagulase in large amounts; Lewis—isolated in April 1948 from a furuncle; known to be hemolytic and to produce coagulase in moderate amounts; 209—Department of Agriculture stock culture used for testing disinfectants; known to be nonhemolytic and a nonproducer of coagulase; and 78—Massachusetts Department of Public Health strain, isolated as a cause of food poisoning; known to be hemolytic and to produce coagulase in small amounts.

One-tenth-ml portions of 18-hour broth subcultures from stock slants were inoculated into 100-ml portions of tryptic digest broth, and the latter was incubated for 1 week at 37 C. The cultures were centrifuged at high speed at 8 C for 45 minutes and the supernatants passed through a Mandler medium filter. The filtrates were assayed by the method cited with the following results: L—hyaluronidase present, the best preparation containing 137 TRU per ml; Lewis—no hyaluronidase; 209—no hyaluronidase; 78—hyaluronidase present, the only 100-ml portion of filtrate assayed showing 40 TRU per ml.

Eleven other strains of *S. aureus* recently isolated from patients, whose histories were unavailable, were assayed qualitatively by the following procedure: Five-ml portions of broth were inoculated from blood agar plate cultures of each strain and incubated at 37 C. The broth supernatant was tested after 24 hours, and again after 48 hours if the 24-hour culture contained no hyaluronidase. Five-tenth-ml portions of the supernatant undiluted, diluted 1:5, and diluted 1:10 with pH 6.0 acetate-chloride buffer were added to 0.5 ml of potassium

hyaluronate solution. Subsequent incubation and turbidity development were followed by the usual quantitative method. The degree of turbidity reduction of each dilution was recorded as: 3 plus—no turbidity; 2 plus—very slight turbidity; 1 plus—moderate turbidity; 0—turbidity equal to that of the usual tube no. 1 in the quantitative method. Of the 11 strains tested, 8 produced no hyaluronidase in 48 hours and 3 produced hyaluronidase in 24 hours.

Schwabacher et al. (1945) studied over 800 strains of staphylococci and micrococci. They found that almost 90 per cent of the coagulase-positive group were also positive for hyaluronidase, as demonstrated by the mucin-clot-prevention test. Most of the deficient organisms were isolated from normal carrier sites or apparently healthy wounds. Of 160 coagulase-negative strains none produced hyaluronidase.

In order to obtain some idea of the time when the enzyme was produced, and of its stability in the medium at 37 C, the following was done: One 100-ml portion of medium was inoculated with the L strain, as previously described. At 48, 96, and 144 hours after inoculation, 5-ml samples were withdrawn and centrifuged, and the supernatant was assayed for hyaluronidase. The results were as follows: 48-hour sample—39 TRU per ml; 96-hour sample—45 TRU per ml; 144-hour sample—75 TRU per ml. These values are only approximations because complete removal of the bacteria from the supernatant was not possible, and as a result there was a slight cloudiness not desirable when a spectrophotometric method is used.

It is of interest that at the end of 1 week replicate cultures inoculated with equal amounts of an apparently homogeneous suspension of the seed cultures and grown under the same conditions sometimes show wide variations in their hyaluronidase content. That this could be due to mutant forms that outgrow the normal forms is suggested by the following observations. At one point in the investigation the stock L strain roughened. Coagulase production was negligible and no hyaluronidase was demonstrated. Reversion to the smooth form with simultaneous satisfactory production of coagulase and hyaluronidase was accomplished by growing the organism in blood broth for a week, transfers being made every 24 hours. Duran-Reynals (1933) reported that extracts of R variants of *S. aureus* with rough colonies contained no spreading factor.

Assays of the L supernatant made immediately before and after filtration show that passage through a Mandler medium filter produces no change in hyaluronidase activity.

Duran-Reynals (1939) recommended extraction with 10 ml of water of a 24-hour agar slant culture, subsequent removal of the bacteria, and the determination of spreading factor present in this extract. Haas (1946) mentioned that hyaluronidase appears in the culture medium during growth of the staphylococcus and that it remains in the solution when the organisms are removed. These references raised the question of whether there is any intracellular hyaluronidase in the staphylococcus, and the following procedure was used in an attempt to provide an answer.

The sediment of an L culture whose supernatant showed the presence of

hyaluronidase was drained of the supernatant as completely as possible, washed with saline solution at 5 C, and centrifuged at 5 C for 30 minutes. The supernatant was discarded and the process repeated. The bacterial sediment was resuspended in approximately 15 times its volume of water, covered with an excess of toluene, and incubated at 37 C for 48 hours. The preparation was then centrifuged at 5 C for 45 minutes and the supernatant autolyzate pipetted from beneath the toluene and passed through a Mandler filter. One ml of the filtrate was diluted with 4 ml of 0.1 M acetate, pH 6.0, containing 0.15 M NaCl, and the mixture assayed. No hyaluronidase was present.

The results indicate that, within the limitations of the assay, there is no intracellular hyaluronidase in the staphylococcus.

Attempted partial purification of hyaluronidase. The literature contains few references to methods for purifying bacterial hyaluronidase. Meyer *et al.* (1940) reported on the precipitation of pneumococcic hyaluronidase by sodium flavianate. Rogers (1948) obtained highly active and purified preparations of streptococcal and staphylococcal hyaluronidase. The bacterial culture medium was mixed with kieselguhr and filtered through paper. It was then dialyzed against tap water for 24 hours in the presence of toluene. After adjustment of the dialyzate to pH 5.6, $Fe(OH)_3$ precipitation was employed. After centrifugation in a Sharples supercentrifuge, the precipitate was eluted with 0.2 M Na_2CO_3, as many as five elutions sometimes being necessary. This procedure gives a 20 to 50 per cent yield.

Because of its relative convenience, Meyer's method was attempted in the present study. The bacterial filtrate was adjusted to pH 3.7 with 1 N H_2SO_4 and centrifuged in the cold after 1 hour at 8 C. For each 20 ml of supernatant 1 ml of 4 per cent sodium flavianate (naphthol yellow S) was added. A yellow precipitate immediately resulted. After centrifugation this precipitate was suspended in water, and 0.01 N NaOH was added drop by drop until solution was just complete. The process was twice repeated. Subsequent assay showed very slight activity:

Crude filtrate	*Flavianate preparation*
16.2 TRU per mg nitrogen	5.0 TRU per mg nitrogen

Possibly this low value can be attributed to denaturation of the enzyme by 0.01 N NaOH and to inadequate control of ionic strength. However, assay of the flavianate supernatant showed hyaluronidase present and assay of the pH 3.7 precipitate showed considerable activity. In view of the latter finding isoelectric precipitation was attempted.

Each of six 20-ml portions of a filtrate obtained from a 6-day culture of the L strain was adjusted to a desired pH by the addition of 1 N H_2SO_4. The pH values were determined by the glass electrode. The pH of the original culture filtrate was 7.8. The first 20-ml portion was adjusted to pH 5.5, the second to pH 5.0, the third to pH 4.5, the fourth to pH 4.0, the fifth to pH 3.5, and the sixth to pH 3.0. All portions were stored at 8 C for 1 hour and then centrifuged in the

cold for 15 minutes. The resulting supernatants were clear. Each sediment was drained of its supernatant and dissolved in 20 ml of Na_2CO_3 solution, pH 7.8, which was made by adjusting the pH of 0.1 M Na_2CO_3 with 0.5 M acetic acid to 7.8. The pH of each sediment was checked by the glass electrode and adjusted to 7.8 with 0.5 M acetic acid when necessary. The dissolved sediments were assayed quantitatively; the supernatant, qualitatively. To rule out the possibility that apparent turbidity reduction of the supernatant might be in reality a failure to produce turbidity, caused by increased ionic strength and an unfavorable pH, an equal amount of unincubated supernatant was tested. This method showed turbidity development comparable to that of the upper blank, in the assay. Thus

TABLE 1

Effect of pH on yield and purity of hyaluronidase

pH	ENZYME UNITS/ML	NITROGEN MG/ML	UNITS/MG NITROGEN	QUALITATIVE TEST OF SUPERNATANT 1/10 DILUTION
7.8—original filtrate	106	2.6	41	+++
5.5—sediment	N. S. Q.	(0.002)	—	+++
5.0—sediment	2.4	0.004	600	+++
4.5—sediment	3.1	0.069	45	+++
4.0—sediment	10	0.067	149	+++
3.5—sediment	11	0.078	141	+++
3.0—sediment	35	0.132	265	+++

it is assumed that turbidity reduction, if present, is due to enzyme action. Nitrogen present in the dissolved sediments was determined by the micro-Kjeldahl digestion method of Wong, followed by Koch-McMeekin nesslerization. The results are shown in table 1.

The highest degree of purification associated with significant yield was 6-fold, at pH 3. The double maximum may be real and may offer confirmation of Rogers' (1948) concept of more than one hyaluronidase. An alternative explanation is that it is only apparent, and caused by the coprecipitation of other materials, particularly procoagulase (Walker et al., 1948), in the pH range 4.5 to 3.5.

SUMMARY

Some strains of *Staphylococcus aureus* produce extracellular hyaluronidase when grown in a tryptic digest medium.

The hyaluronidase-positive L strain apparently contains no intracellular hyaluronidase.

The R mutant of the hyaluronidase-positive L strain fails to produce hyal-

uronidase.   Hyaluronidase production is resumed upon reversion to the normal
S form.

Staphylococcal hyaluronidase can be concentrated and purified to some extent
by precipitation at pH 3.0.

The present evidence may indicate the existence of more than one staphylo-
coccal hyaluronidase, as suggested by Rogers (1948).

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THE METABOLISM OF SPECIES OF STREPTOMYCES

I. The Formation of Succinic and Other Acids[1]

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That certain species of *Streptomyces* produce acids during growth in culture has been known for almost fifty years, Beijerinck (1900) having noted the formation of acid, "probably lactic acid," by an isolate belonging to this genus. Data from several workers, e.g., Krainsky (1914), Ørskov (1938), Kelner and Morton (1947), and Johnstone and Waksman (1948), indicate that acids are produced by many forms in carbohydrate media. Von Plotho (1940) reported four strains as forming lactic acid, relying on color tests and the appearance of the calcium salt for identification. The same author found no evidence of succinic acid production in his glucose nutrient broth medium.

Lactic acid has been positively identified as a minor product of the glucose metabolism of *Streptomyces lavendulae* (Woodruff and Foster, 1943) and *Streptomyces griseus* (Dulaney and Perlman, 1947).

In the original description of *Streptomyces coelicolor*, Müller (1908) stated that it does not form acid on litmus ascites agar with glucose, and this characterization of the species has remained in the taxonomic literature. Conn and Conn (1941) showed that acid is formed from glucose by a strain identical in all other respects with that described by Müller. The bulk of the present work is an analysis of acid production by this strain, with the demonstration that the major acid produced is succinic acid. In addition, comparative studies of two other species are reported.

METHODS

The organisms investigated were *Streptomyces coelicolor* (Müller) Waksman and Henrici, isolate B-3 of Conn (1943); *Streptomyces griseus* (Krainsky) Waksman and Henrici, strain no. 4; *Streptomyces reticuli* (Waksman and Curtis) Waksman and Henrici; and an unnamed isolate carried in this laboratory as A-105.

Except as noted, the growth medium contained cp glucose monohydrate, 0.05 M; Difco asparagine, 0.0075 M; cp K_2HPO_4, 0.003 M; cp $MgSO_4 \cdot 7H_2O$, 0.001 M; Difco yeast extract 0.25 g per L; and a minor element mixture supplying Fe, Zn, Cu, Mn, Mo, and B. Sterile $CaCO_3$, 0.3 per cent, was added after autoclaving. The medium is a slight modification of that used in earlier work (Cochrane and Conn, 1947), the changes having been made to give maximum growth rates in aerated culture.

We have found it extremely important that the major constituents of culture media for actinomycetes be in balance. "Balance" means that absolute and rela-

tive concentrations of glucose and asparagine are so chosen that glucose utilization is rapid and complete in a reasonable time and that asparagine (or other nitrogen source) is added at only the optimum level for growth. An excess of either main constituent results in derangement of the metabolic pattern. Perhaps the most common error in cultivating actinomycetes is the use of media high in carbohydrate and low in utilizable nitrogen. The nitrogen requirement of the group is generally low, but it is not negligible.

The organism was grown in 500-ml Erlenmeyer flasks, each containing 100 ml of medium and mounted on a reciprocating shaker having a speed of 96 cycles per minute and a stroke length of 9 cm. The temperature of incubation was 24 to 25 C. Under these conditions sugar utilization by S. coelicolor is 90 per cent complete in 4 days, as compared to 18 days required by the same organism on the same medium in still culture.

All acid determinations were made on an ether extract of the culture filtrate. The filtrate was adjusted to pH 1.5 with H_2SO_4 and extracted 18 hours with freshly purified ether in a Kutscher-Steudel extractor. Recovery of added succinic acid was complete, that of added lactic acid 97 to 99 per cent complete. Data on total organic acids and lactic acid are corrected for recovery and for amounts present in the sterile culture medium. The total organic acid concentration was determined by electrometric titration over the range pH 7.8 to 2.6. Lactic acid was estimated by the permanganate oxidation method of Edwards (1938), and succinic acid by precipitation of the silver salt and volumetric determination of silver in the precipitate (Phelps et al., 1939). Fumaric acid was estimated by precipitation of the insoluble mercurous fumarate (Stotz, 1937). Volatile acids were determined by titration after a 10-volume steam distillation at pH 2.0.

Sugar was determined by the method of Somogyi (1945), and pH by the glass electrode. Mycelial growth in terms of dry weight per flask was determined by filtering the culture through a tared filter paper. The material on the paper was washed with 1 per cent HCl to remove the carbonate, rinsed, and dried to constant weight at 105 C. An error not exceeding 1 per cent is introduced through absorption of atmospheric moisture by the filter paper during weighing. Reported weights represent the average of 4 replicates.

RESULTS

The acids produced by S. coelicolor. Preliminary experiments demonstrated that unexpectedly large amounts of acid are formed by this organism in a glucose medium. Yields of total organic acid as high as 30 milliequivalents per L are not uncommon, and the amount may be increased by raising the glucose concentration of the medium.

Solubility and color tests, paper chromatography, and distillation data excluded from consideration the common metabolic products formic, acetic, propionic, butyric, glycolic, oxalic, gluconic, malic, and citric acids.

Analyses made it evident that only a small fraction of the total acid can be accounted for as lactic acid. The remainder, from the solubility of its barium salt, appeared to be one or more dicarboxylic acids.

The organism was grown 6 days on the standard medium lacking yeast extract, and 1,500 ml of the culture fluid was separated by filtration. This was evaporated to 50 ml, acidified to pH 1.5, and extracted with ether. The ether extract was taken up in water, excess neutral lead acetate was added, and the preparation was allowed to stand overnight at 5 C. The lead salt was removed by centrifugation, washed twice with 80 per cent alcohol, and decomposed with H_2S. The total acid remaining was 8.5 milliequivalents. The acid was neutralized with Na_2CO_3 and refluxed 2 hours with p-phenylphenacyl bromide in 65 per cent alcohol. The ester that separated was twice recrystallized from acetone, and it melted at 207 to 208 C (theoretical for di-p-phenylphenacyl succinate 208 C).

To eliminate the possibility that the succinate might have arisen by reductive deamination of aspartic acid (Gale, 1947), a second preparation was made from cells grown on a medium in which the asparagine was replaced by $(NH_4)_2CO_3$ (0.0075 M). The same isolation procedure yielded 5.3 milliequivalents of the unknown acid from 1,600 ml of culture filtrate. From this the p-bromphenacyl ester was prepared and found to melt at 211 to 213 C (theoretical for the succinic ester 212 C). A mixed melting point with authentic di-p-bromphenacyl succinate showed no depression. It is concluded therefore that the succinic acid found is formed from glucose.

In a final preparation the same procedure was followed up to the decomposition of the lead salt. The free acid was treated with excess $KMnO_4$ in acid solution, extracted with ether at pH 9, and steam-distilled to remove impurities. Acid ether extraction was followed by precipitation of the free acid from boiling ethyl ether with petroleum ether. The crystals so isolated, after recrystallization, melted at 184 to 185 C (theoretical for succinic acid 185 C).

In addition, the presence of a small amount of fumaric acid, not over 3 per cent of the succinate present, was indicated by an analysis of the dicarboxylic acid fraction using the mercurous fumarate method of Stotz (1937). It should be noted that some contamination with mercurous succinate is a possibility.

Traces of another acid were also present in the lead preparation, and a semicarbazone melting at 248 to 250 C was isolated. This suggests that there may be a very small amount of a keto acid present, not *alpha*-ketoglutaric or pyruvic. Application of the "total keto-acid" method of Friedemann and Haugen (1943) confirmed this qualitatively, but the amounts present were too small for accuracy.

S. coelicolor thus produces from glucose succinic acid, lactic acid, and possibly small amounts of fumaric and an unidentified keto acid. Volatile acids are not present or are present in negligible amounts (not over 0.5 per cent of the total organic acidity).

Comparative acid production by Streptomyces species. In table 1 are displayed comparative data on several species, grown under similar conditions but at different times. It is clear that *S. coelicolor* differs sharply from the other forms. The total production of succinic acid by *S. coelicolor* amounts to about 0.18 moles per mole of glucose consumed. Digestion of the mycelium with dilute mineral acid liberates an additional amount of organic acid equivalent to 10 per cent of that in the filtrate. Inclusion of this fraction raises the ratio above to approximately 0.20 moles succinate per mole of glucose consumed.

Under the conditions specified, succinic acid accounts for 93.5 per cent of the total organic acids, lactic acid for only 7.6 per cent. This relation may, however, be modified profoundly by alterations in the conditions of culture, although it is relatively uniform in the absence of such alteration.

Volatile acids were absent or negligible in amount in all cultures, and the lanthanum nitrate color test for acetic and propionic acids (Feigl, 1939) was negative. A particular effort was made to locate volatile acids because of their known formation by an anaerobic *Micromonospora* (Hungate, 1946) and their possible phylogenetic significance in strengthening the link between the actinomycetes and the propionic acid bacteria. It seems clear that under the highly aerobic conditions used, volatile acids do not accumulate. The traces of titratable volatile acidity found never over 0.1 m.e. per L, conceivably represent the low but appreciable volatility in steam of lactic acid.

TABLE 1

*Comparative growth and acid formation of Streptomyces species**

	S. COELICOLOR	S. GRISEUS	S. RETICULI	S. SP. 105
Dry weight, mg per 100 ml......................	132.0	101.3	114.6	58.8
Residual glucose, mg per 100 ml................	37.5	62.4	17.3	23.3
Glucose consumed, moles per L..................	0.048	0.047	0.049	0.049
Total organic acid, m.e. per L..................	18.59	0.55	0.62	1.69
Succinic acid, m.e. per L.......................	17.39	0.09	0.00	0.00
Lactic acid, m.e. per L.........................	1.42	0.38	0.20	1.33
Initial pH......................................	6.96	6.99	6.96	6.98
Terminal pH....................................	8.08	6.89	7.71	8.27

* Medium: glucose, 0.05 M; asparagine, 0.0075 M; K_2HPO_4, 0.003 M; $MgSO_4 \cdot 7H_2O$, 0.001 M; yeast extract, 0.25 g per L; $CaCO_3$, 3.0 g per L; and minor elements. Reciprocating shaker, 24 to 25 C. Period of growth, 6 days.

By comparison, the other strains shown in table 1 produce very little acid of any type, and only one of these formed even traces of succinic acid. The formation of succinate by *S. griseus* is discussed below. In *S. griseus*, *S. reticuli*, and *Streptomyces* sp. A-105, the total acidity is approximately accounted for by the lactic acid found, and there is no evidence that the discrepancies between total acid and lactic acid represent anything other than analytical difficulties in dealing with rather small quantities.

The course of metabolism in S. coelicolor. In figure 1 are plotted data on the temporal course of growth, glucose utilization, and acid formation by *S. coelicolor*. In general, sugar utilization is accompanied by an increase in dry weight, a steady decline in pH, and rapid accumulation of succinic acid. The accumulation of succinate begins on the third day, when approximately 75 per cent of the sugar has been consumed, and reaches its maximum on the sixth day, although the change after the fifth day is not large. These data implicate succinate as a direct product of glucose metabolism. If it is being metabolized, i.e., if the accumulation measures an equilibrium between formation and breakdown of

succinate, it seems clear from the net gain in succinate between the third and the fifth days that glucose is being consumed at a much faster rate than the acid.

Actually there is little evidence that succinate is in fact broken down by the organism at any appreciable rate. The decline in succinic acid after the glucose has disappeared is, in this and all confirming experiments, very slow. As a sole source of carbon, succinate is utilized very slowly by *S. coelicolor*.
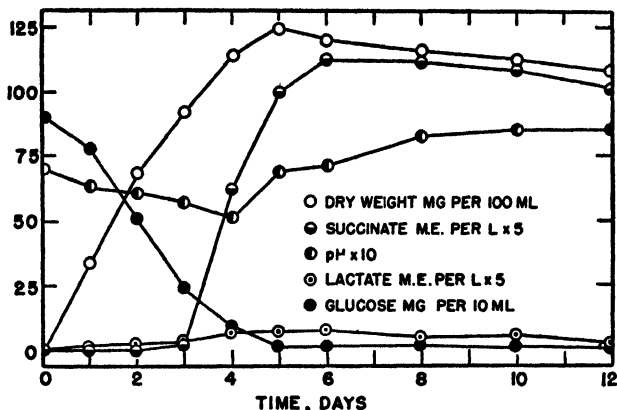
Figure 1. Metabolism of *Streptomyces coelicolor*. Medium: glucose, 0.05 M; asparagine, 0.0075 M; K_2HPO_4, 0.003 M; $MgSO_4 \cdot 7H_2O$, 0.001 M; yeast extract, 0.25 g per L; $CaCO_3$, 3.0 g per L; and minor elements. Reciprocating shaker, 24 to 25 C.
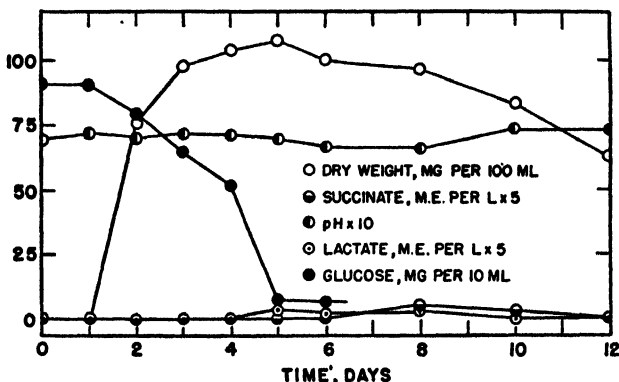
Figure 2. Metabolism of *Streptomyces griseus*. Medium and conditions as in figure 1.

The pH rise after the fourth day shown in figure 1 is thus not the result of acid breakdown. Presumably the release of ammonia from asparagine or from autolyzing cells accounts for the increased alkalinity.

The course of lactic acid accumulation is more indicative of an equilibrium between formation and utilization, but the level of accumulation is so low that definite conclusions are impossible.

The course of metabolism in S. griseus. The curves for this species (figure 2) differ most from those for *S. coelicolor* in that acids are not a significant product

at any time. Correspondingly, there is no very sharp drop in pH. The rates of growth and sugar utilization are very similar to those of S. coelicolor.

Both lactic and succinic acids are formed, but neither accumulates to any appreciable extent. It is also true, in contrast to S. coelicolor, that the first appearance of succinate in the medium is a full day after the glucose has essentially disappeared. Under these circumstances, with autolysis already under way, it is difficult to make any suggestions regarding the metabolic origin of the traces of succinate found.

The course of metabolism in S. reticuli. The data for this species need not be presented in detail, since with few exceptions the course of metabolism is similar to that of S. griseus. The difference largely resides in the rapid growth rate of S. reticuli, which reaches its peak dry weight in 3 days and autolyzes rapidly thereafter. So far as acid accumulation is concerned, the general pattern is the same as that of S. griseus.

Genetic stability of biochemical properties. In preparation for a study of radiation-induced mutants of the organisms whose metabolic cycles have been described, some preliminary investigation has been made of the stability of the biochemical pattern in regard to the accumulation or nonaccumulation of acids from glucose. The method used was to plate out cultures on the indicator agar medium described previously (Cochrane, 1947). Of 220 colonies (presumably arising from single spores) of S. coelicolor, all produced acid within 3 days. Of 268 colonies of S. reticuli, none formed acid in 7 days. These results are of interest primarily because of the prevalent view that the characters of the actinomycetes are much more unstable than is usual among microorganisms. We have not found any instability in pigment production (Cochrane and Conn, 1947), and the foregoing preliminary data suggest that spontaneous loss or gain of ability to form acids from glucose is at least not common.

DISCUSSION

The present report adds the actinomycetes to the groups known to produce succinic acid. Quantitatively the amount of succinate formed by S. coelicolor, 0.2 moles per mole of glucose consumed, may be compared with the corresponding figures of 0.4 for *Escherichia coli* (Stokes, 1949), 0.05 for resting yeast cells (Weinhouse et al., 1948), and 0.16 for *Pasteurella pestis* (Doudoroff, 1943).

No conclusions as to the mechanism of succinate formation by S. coelicolor can be reached from the data here presented. It should be noted that all cultures contained excess $CaCO_3$.

Within the actinomycetes, taxonomic separation is notoriously uncertain at the present time. As information accumulates on the fundamental metabolic processes of these organisms, the difficulties will undoubtedly lessen. No one character will be adequate; it has already been shown (Cochrane, 1947) that the criterion of acid production versus failure of such production characterizes satisfactorily only about 70 per cent of a group of 100 isolates. Comparison of the detailed data presented herein leaves no doubt that S. coelicolor represents a type of metabolism clearly differentiated from that of S. reticuli, and that the differ-

ences between *S. reticuli* and *S. griseus*, though less marked, are easily definable.

Superficially, it might seem that succinate formation by *S. coelicolor*, a filamentous and strictly aerobic organism, might be comparable to organic acid accumulation by fungi. Actually, most of the syntheses by fungi take place, as Foster (1947) has pointed out, in media in which a great excess of carbohydrate is present. Under such circumstances the concept of a "shunt" or "overflow" mechanism may be applicable. In the present instance two lines of evidence militate against any such interpretation. First, the medium was so designed as not to contain an excess of either the carbon or the nitrogen source over that required for growth. Second and more critical, the data (figure 1) show that succinate accumulation begins actively before the glucose is exhausted and, further, that even after the disappearance of glucose the succinate present is not metabolized at an appreciable rate by the presumably starving cells.

A more likely hypothesis is that succinate is a normal transient metabolite of the "typical" actinomycete, represented here by *S. griseus* and *S. reticuli*. *S. coelicolor*, on this hypothesis, is a form in which, as a result of genetic change in the past, the enzyme system responsible for the conversion of succinate into other compounds has been lost or severely impaired in its function.

SUMMARY

Streptomyces coelicolor produces, in a glucose medium with excess $CaCO_3$, relatively large amounts of succinic acid, small amounts of lactic acid, and traces of fumaric acid and an unidentified keto acid. Volatile acids were not detected. Evidence is presented that succinic acid is formed during active glucose consumption and that it is formed in media with a variety of nitrogen sources.

By comparison, *Streptomyces griseus*, *Streptomyces reticuli*, and an unnamed isolate form very little acid from glucose and no succinic acid during active growth. Traces of succinate appear in late stages of cultures of *S. griseus*.

Differences between species in acid formation and in growth rates under standardized conditions may be added to pigment production as stable characters that may find application in species separation.

It is suggested, from the evidence at hand, that *S. coelicolor* represents a genetic change from the usual actinomycete metabolic pattern, a change resulting in loss or impairment of the enzyme systems concerned with the metabolism of organic acids.

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THE DETERMINATION OF LYSOZYME[1]

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Since the discovery of lysozyme by Fleming (1922) several authors have proposed different methods for determining the activity of various preparations containing this enzyme. Most of these methods were based upon the clearing of dense suspensions of a susceptible organism without concern for accurate quantitative results. The isolation of highly purified crystalline lysozyme by Alderton and Fevold (1946) suggested the possibility of a method for assaying lysozyme. The procedure described here provides for the rapid and accurate microbiological assay of materials that show lytic activity considered to be due to lysozyme.

Fleming (1922), in his original work, observed the lytic activity of lysozyme either as clear zones on agar plates seeded with *Micrococcus lysodeikticus* or as a clearing of a suspension of the same organism. Sandow (1926) used serial dilutions of egg white in meat infusion broth inoculated with various species of organisms. After incubation these mixtures were observed for growth, as evidenced by the turbidity of the tubes. This procedure was applied to a study of the different organisms affected by lysozyme, the dilution of egg white capable of producing inhibition or sterilization being noted.

Goldsworthy and Florey (1930) devised a scheme of assay which consisted of washing an 18-hour culture of *M. lysodeikticus* with saline and adjusting the opacity of that suspension to that of Brown's barium sulfate standard no. 4. Lysozyme was serially diluted so that each succeeding dilution contained only half as much of the enzyme as the one previous. Then 0.5 ml of each dilution was mixed with an equal quantity of cell suspension. The mixtures were allowed to incubate at 38 C for 1 hour. A unit was defined as the least amount of lysozyme necessary to produce complete lysis.

Rosenthal and Lieberman (1931) in determining the lysozyme content of infant stools mixed a susceptible sarcina with stool extracts. Visual observations of the mixture were made under the microscope. A disappearance of the sarcina cells indicated lysozyme activity.

Boasson (1938) developed a technique involving the use of optical measurements of turbidity. A phenol-killed suspension of the test organism was mixed with various dilutions of lysozyme. The amount and rate of clearing was carefully measured in a Moll extinctometer and correlated with the concentration of lysozyme. Since the activity for the known concentration could be observed accurately, it was possible to compare the extent of activity of an unknown and in this manner to determine the amount of lysozyme present.

The method herein reported is somewhat similar to the Boasson technique and to the one employed by Goldsworthy and Florey (1930).

Meyer and Hahnel (1946) developed a viscosimetric method for measuring the mucolytic activity of lysozyme. A mucopolysaccharide was used as a substrate for the enzyme. The test was based on the depolymerization of this material, which thereby effected a change in the viscosity of the substrate-enzyme mixture. The change in viscosity could be measured and correlated with the lysozyme concentration. The preparation of this mucopolysaccharide fraction is a very involved and laborious procedure, which does not lend itself to a rapid method of assay.

Meyer, Hahnel, and Steinberg (1946), in reporting on lysozyme of plant origin, used the viscosimetric method to measure mucolytic activity and an optical method to measure the bacteriolytic activity. The optical method was not very different from those used by other investigators. *M. lysodeikticus* cells were suspended in a M/15 phosphate solution and mixed with lysozyme dilutions. After 1 hour of incubation at 37 C, 2 drops of normal sodium hydroxide were added and the clearing was read visually. A unit was expressed as the highest dilution giving complete visible clearing under the conditions specified.

Hartsell (1948) used a suspension of *M. lysodeikticus* in phosphate buffer, pH 6.2, mixed with lysozyme contained in rehydrated, spray-dried, whole egg powder. An incubation temperature of 52 C was used, and clearing of the suspension was observed visually. The lysozyme concentration in a sample was expressed as the reciprocal of the highest dilution causing clearing.

None of the tests described above appear to be capable of providing accurate results with a minimum expenditure of time. The desire for an accurate and rapid method of assay prompted this study.

METHOD

The method for lysozyme assay described here is based on a comparison of light transmissions of crystalline lysozyme dilutions with the values for the substance being tested, after the addition of susceptible cells and incubation. A purified chloride salt of lysozyme[2] is used as the standard. This material was chosen because of its availability and constancy of activity.

M. lysodeikticus Fleming, ATCC 4698, is subcultured on yeast water, veal infusion agar[3] with 0.2 per cent glucose every 24 hours for 3 days. After the final subculturing a suspension of the organism is made in phosphate buffer, pH 6.2, and a large number of Roux bottles containing the same medium are inoculated. A heavy suspension as inoculum with a minimum amount of residual moisture on the surface of the agar will give the highest yield of cells. After 18 hours at 37 C the cells are harvested in phosphate buffer. The suspension is then exposed to ultraviolet light in the following manner: A "mediquartz"

[2] The crystalline lysozyme was supplied by the Western Regional Research Laboratory, U.S.D.A., Albany, California, and The Armour Laboratories, Chicago 9, Illinois.

[3] Ten per cent veal infusion, 10 per cent yeast water, 0.5 per cent salt, 0.5 per cent peptone, and 2 per cent agar.

germicidal lamp is placed about 4 inches above an inclined piece of Venetian glass. The cell suspension is allowed to flow slowly down the rough side of the glass. The procedure is repeated. In this manner the cells are twice exposed to approximately 2,700 microwatts per cm², [4] which provides a cell suspension with but few living cells. The organisms are then collected, shell-frozen, and dried in a vacuum. In this way it is possible to obtain approximately 0.15 g of dried cells per 100 ml of medium. The cells are stored at 4 C until needed.

The cell suspension is prepared from the dried cells in Sørenson's phosphate buffer, pH 6.2, prior to the assay. Rehydration is easily accomplished since lyophilized cells can be readily resuspended. The turbidity of this suspension is adjusted to show 10 per cent light transmission in a Coleman spectrophotometer equipped with a PC-4 filter, at a wave length of 540 mμ, and to show 100 per cent transmission with a distilled water blank.

From a 1:10,000 stock solution of crystalline lysozyme, dilutions are prepared in Sørenson's phosphate buffer, pH 6.2. Twofold dilutions, starting with 1:200,000 and progressing to 1:6,400,000, are made, thus giving a range of 0.0007 to 0.025 mg of lysozyme per 5 ml of dilution used. Prior to the test the unknown should be checked for its activity to determine what dilutions are needed to give the same level of activity as the crystalline control.

Before the assay is begun a sufficient number of test tubes are matched so that all tubes will show the same light transmission value with distilled water as a reference. At measured intervals a 5-ml quantity of the lysozyme dilution is mixed with 5 ml of the cell suspension. The same procedure is used for the dilutions of the material being tested. All mixtures are made in duplicate. After 20 minutes' incubation at room temperature, the light transmissions for the various mixtures are recorded and the concentration of the unknown is determined. The results of a typical test are shown in table 1.

With the transmission values for the crystalline lysozyme mixtures, a standard curve is prepared by plotting the transmission against concentration. A log scale is used on the abscissa of the standard curve. The transmission values for the unknown dilutions are located on the ordinate and projected to the standard curve. By projection to the abscissa from these points, the concentration of lysozyme in each dilution is determined. Multiplication by the dilution factor results in the concentration of lysozyme per ml of undiluted extract. Figure 1 represents the curve for the values in table 1.

Among the criteria of adequacy of a test of this nature are the accuracy and reliability of the results. The effectiveness of this test was established in a series of assays using materials that showed lytic activity and were capable of maintaining this power when stored. The first preparation tested was powdered egg albumin, which upon rehydration in phosphate buffer showed high lytic activity. The results of the assay of this material are given in table 2. It is to be noted that the titers are consistent and that the error does not exceed 7 per cent, [5] which is not considered excessive for this type of assay. Some of the

[4] All wave lengths are less than 3,400 A.
[5] Computed from the *A.S.T.M. Manual on Presentation of Data*, Supplement A., A.S.T.M., July, 1947, the data indicate 99 per cent certainty that the error does not exceed 7 per cent.

differences in the values may be attributed to the difficulty of uniform rehydration of the albumin.

TABLE 1

Assay results for rat kidney extract

DILUTION	AVG % T	2-LOG % T	LYSOZYME MG PER ML	
			Diluted* extract	Undiluted† extract
Rat kidney extract				
1:10	68.7	0.1627	0.0021	0.021
1:20	56	0.2518	0.0013	0.026
1:40	41	0.387	0.0006	0.024
1:60	34	0.469	0.0004	0.024
Crystalline control				
1:200,000	82.3	0.0848		
1:400,000	73.7	0.1322		
1:800,000	55	0.2596		
1:1,600,000	40.3	0.395		
1:3,200,000	31.3	0.505		
1:6,400,000	27	0.569		

* Value obtained from figure 1.
† Value obtained by multiplying the amount obtained from figure 1 by the dilution factor.
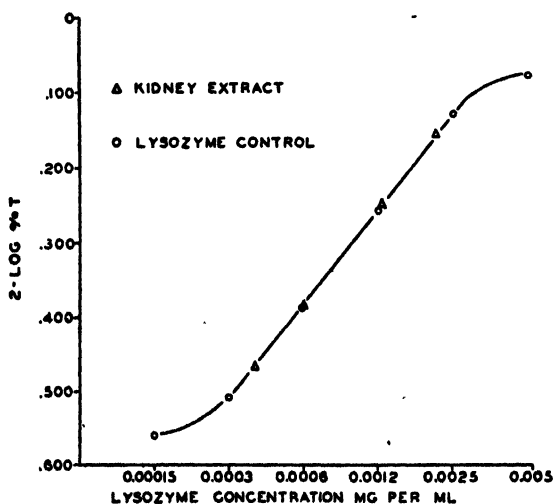
Figure 1. The result of a typical assay with rat kidney extract.

Other preparations with a lower lysozyme concentration were tested by the same method. A rat kidney extract and a hen's egg albumin extract[6] provided

[6] Alderton, Ward, and Fevold (1945) bentonite pyridine extraction, as modified by H. Feldmann, Purdue University; personal communication.

further evidence for the reliability of the test. These results are given in tables 3 and 4. The values are not absolute titers because the extracts were not prepared for quantitative determinations. The rat kidney and the albumin extract both show the same reliability of titers during storage in the icebox for a period of 4 weeks.

TABLE 2

Lysozyme titers of dried egg albumin rehydrated in phosphate buffer, pH 6.2

ASSAY NO.	LYSOZYME	ASSAY NO.	LYSOZYME
	mg per g		*mg per g*
1	20.3	9	22.1
2	19.2	10	20.2
3	19.8	11	19.1
4	20.1	12	16.6
5	20.4	13	18.8
6	21.1	14	16.0
7	21.1	15	17.7
8	20.9	16	21.9

TABLE 3

Lysozyme titers of hen's egg albumin extract

ASSAY NO.	LYSOZYME	ASSAY NO.	LYSOZYME
	mg per ml		*mg per ml*
1	0.80	5	0.85
2	0.79	6	0.87
3	0.89	7	0.91
4	0.91	8	0.91

TABLE 4

Lysozyme titers of rat kidney extract

ASSAY NO.	LYSOZYME	ASSAY NO.	LYSOZYME
	mg per ml		*mg per ml*
1	0.023	5	0.026
2	0.024	6	0.023
3	0.025	7	0.025
4	0.022		

To further demonstrate the practicability of this test several students conducted the assay using the same source of unknown material. The results were similar and the differences were within the limits of experimental error.

SUMMARY

The method of assay for lysozyme described has proved to be sufficiently accurate to recommend its use in testing various materials for lytic activity.

It has been successfully applied to the examination of several preparations containing lysozyme (dried egg albumin, fresh hen's egg albumin, and extracts from animal tissue). The method is rapid and thus makes possible the testing of large numbers of materials in a relatively short time.

A method has been described for the preparation of a large number of *Micrococcus lysodeikticus* cells for use in the turbidimetric assay of lysozyme. It is possible to store these cells at icebox temperatures without any appreciable reduction in their sensitivity when used in assaying lysozyme. Consistent titers for lysozyme activity were obtained in replicate tests.

The method has been shown to be adaptable for both high and low concentrations of the enzyme from natural materials, and gives reproducible results in each instance.

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FACTORS AFFECTING RIBOFLAVIN PRODUCTION BY ASHBYA GOSSYPII[1]

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The methods that are presently employed for the microbiological production of riboflavin on a commercial scale are based upon the remarkable biosynthetic capacities of two species, viz., *Clostridium acetobutylicum* and *Eremothecium ashbyii*, for this vitamin. With *C. acetobutylicum* riboflavin appears concurrently during the anaerobic fermentation of various carbohydrates to butanol, acetone, and ethanol. As early as 1927 Weyer and Rettger observed that a yellow pigment accumulates in cereal mashes fermented by this organism. They attributed it to zein dissolved from corn by the solvent. It was not until several years later (Yamasaki and Yositome, 1938; Miner, 1940) that this pigmented substance was identified as riboflavin. Subsequent studies on the butyl anaerobes by Arzberger (1943), Meade *et al.* (1945), and others have revealed the critical relationship that exists between the iron concentration of the medium and riboflavin biosynthesis. By controlling this variable, either through adding or restricting iron as dictated by the materials fermented, processes have been developed that are applicable to whey and cereal grain mashes.

The production of a greenish-yellow pigment by *Eremothecium ashbyii* cultivated upon certain solid media was reported in 1935 by Guilliermond *et al.* The identity of this substance with riboflavin was established by the later studies of Raffy (1937) and Mirimanoff and Raffy (1938a,b). Under submerged aerobic conditions unusually high concentrations of riboflavin are formed by *E. ashbyii*, and most of the biologically produced vitamin is made with this organism. Several processes involving *E. ashbyii* combined with specific media and submerged aerobic techniques have been patented within the past few years (Rudert, 1945; Piersma, 1946; Mayer, 1946; Foster, 1947).

In addition to the two organisms described above, the elaboration of substantial amounts of riboflavin by other yeasts and bacteria is not uncommon. Examples of such include yeasts of the genus *Candida* (Burkholder, 1943; Tanner *et al.*, 1945), *Mycobacterium smegmatis* (Mayer and Rodbart, 1946), and *Aerobacter aerogenes* (Novak, 1948). Because these species synthesize lesser amounts of riboflavin or because they require carefully controlled environments

for maximum production, they hold less promise of industrial utilization than the two types now in commercial use.

Guilliermond *et al.* (1935) observed that under the conditions suitable for riboflavin accumulation by *Eremothecium ashbyii*, the closely related species *Ashbya gossypii* formed only traces of this vitamin. Confirmation of this feature distinguishing the two species may be found also in the reports of Schopfer (1944) and Deseive (1947). Nevertheless, Wickerham *et al.* (1946) found that *A. gossypii* was capable of substantial synthesis of riboflavin when selected media and cultural conditions were provided. For example, a medium composed of glucose, Difco peptone, and Difco yeast extract inoculated with *A. gossypii* and aerated by introducing sterile air gave culture liquors within an incubation period of 8 days which assayed as high as 381 μg per ml. These yields, although somewhat below those reported for *E. ashbyii*, indicated the industrial potentialities of this organism and justified a more intensive study.

In the present investigation the effects of various factors upon riboflavin synthesis by *A. gossypii* have been investigated. Particular attention has been paid to available low-cost media, cultural conditions, inoculum development, and sterilization procedures, since these were found to have an important bearing upon the riboflavin productivity of this organism. Pilot-plant studies have been undertaken utilizing the more favorable conditions developed herein, and these will be reported separately.

METHODS

At the beginning of this study, *A. gossypii* was maintained on agar slants containing 1 per cent glucose, 0.5 per cent peptone, 0.3 per cent yeast extract, and 1.8 per cent agar. Later this medium was supplemented with 0.3 per cent Difco malt extract. Malt extract appeared to stimulate the rate of pigment formation on agar media, but there was no evidence that this beneficial influence was transmitted to the liquid media subsequently used in the principal fermentation.

To study riboflavin biosynthesis in submerged fermentations, liquid media was dispensed in 100-ml amounts in 500-ml Erlenmeyer flasks. These were autoclaved 15 minutes at 121 C, cooled, and inoculated with a liquid culture propagated under submerged aerobic conditions. All flasks were incubated at 28 $\pm$ 1 C on a reciprocating shaker having 92 three-inch strokes per minute. At suitable intervals, samples were withdrawn for routine analyses. Riboflavin was determined with a photofluorometer after hydrolysis of the sample by autoclaving for 30 minutes either in the presence of 0.1 N HCl or 0.123 M acetate buffer, pH 4.7. Unlike many natural materials, the riboflavin in *A. gossypii* cultures was readily liberated by water extraction. However, the acid or buffer provided the necessary stabilization of riboflavin during the hydrolysis of culture liquors which had reached a definitely alkaline reaction. Fluorometric analyses compared very well with those obtained microbiologically and spectrophotometrically.

Several factors that modify riboflavin synthesis were studied in turn and more or less independently, and accordingly the importance of each factor was not fully appreciated at the time some of the data were recorded. Especially was this true during the early part of the investigation before it was fully recognized that a number of conditions were capable of deleteriously affecting growth or riboflavin production. Some of these are pointed out below; others are as yet incompletely understood. Consequently, most of the tabular data are indicative primarily of the significance of a particular variable rather than of the maximum yield obtainable under each set of conditions.

Temperature. Although *A. gossypii* grows abundantly over a rather wide temperature range, the greatest riboflavin accumulation occurred within fairly
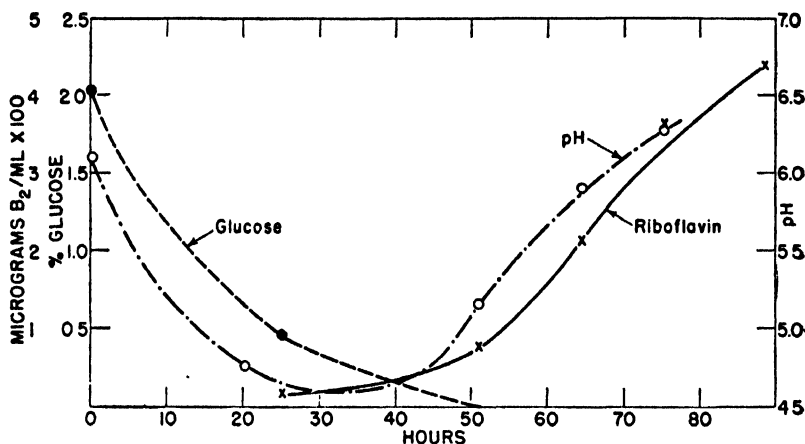
Figure 1. The fermentor was charged with 2,470 grams of corn steep liquor, 617.5 grams of Difco peptone, 5,000 grams of glucose monohydrate, and approximately 51 gallons of tap water. The pH was adjusted to 6.57 with sodium hydroxide before sterilization. The medium was sterilized by injecting steam and maintaining a pressure of 15 pounds for 35 minutes. After cooling, to 28 C, the medium received 6 liters of 24-hour inoculum and was aerated at a rate of one-fourth volume of air per volume of medium per minute.

narrow limits. The highest yields were obtained when cultures were incubated at 26 to 28 C, which is below the optimum for growth. Incubation at temperatures higher than 28 C resulted in sharply decreased yields, whereas low temperatures were less critical but required longer fermentation periods to obtain equivalent potencies.

Reaction of the medium. The best yields of riboflavin were obtained when the initial reaction of the medium was above pH 5.5, and preferably in the range of pH 6.0 to 7.0. When the initial reaction was between pH 4.5 and 5.5, good and rapid multiplication followed, but the amount of riboflavin produced was less than when the foregoing preferred range was used. Media initially adjusted to pH 4.0 gave little growth or.riboflavin.

Data from a tank fermentation which are presented in figure 1 illustrated

the course of a typical fermentation. It may be seen that the fermentation is comprised of two rather distinct phases. In the first, glucose is dissimilated and the medium becomes acid, generally reaching about pH 4.7 with an occasional minimum of pH 4.5. This change occurs within the first 24 to 36 hours. When glucose consumption is substantially complete, there is a gradual rise to an alkaline reaction occasionally reaching pH 8.5. Only negligible amounts of riboflavin appear in the medium before the carbohydrate is metabolized, the bulk of it being formed during the second phase in which a neutral or alkaline reaction develops. Fermentations in shaken flasks follow a similar pattern, but generally require longer incubation periods, probably owing to less efficient aeration under such conditions.

TABLE 1

Riboflavin synthesis by Ashbya gossypii* in relation to initial glucose supply

INITIAL GLUCOSE	pH			RIBOFLAVIN		
	4 days	6 days	9 days	4 days	6 days	9 days
per cent				*µg/ml*		
0	8.0	8.1	8.2	0.4	0.4	0.5
0.25	8.3	8.3	8.5	10.2	10.5	11.7
0.50	8.3	8.3	8.2	23	23	23
1.0	8.1	8.1	8.3	35	35	34
2.0	6.8	8.2	8.3	125	159	274
3.0	6.1	6.8	8.4	210	340	365
4.0	6.2	6.2	7.7	297	339	352
5.0	5.4	6.2	6.1	208	301	344

* Flasks inoculated with 1.0 per cent of 24-hour liquid culture grown on 2.0 per cent glucose, 0.5 per cent peptone, and 0.3 per cent yeast extract.

Carbohydrate sources and concentration. The amount of riboflavin formed was found to be closely related, within certain limits, to the quantity of fermentable sugar supplied; in a basal medium containing 0.5 per cent corn steep liquor and from 0.25 to 3.0 per cent glucose, riboflavin appeared to increase with the amount of sugar supplied. The difference between 3.0 and 4.0 per cent glucose was slight, and levels above 4.0 per cent were not beneficial although 10 per cent sugar was found in other trials to have no detrimental effect. When sugar was supplied in concentrations below 0.25 per cent, apparently it was utilized largely for growth and an alkaline reaction developed rapidly without appreciable riboflavin production. When the available carbohydrate was increased, the characteristic pH changes took place and were accompanied by a much greater synthesis of riboflavin.

A. gossypii possesses little or no saccharifying power; thus, starch or modified starches fail to serve adequately as carbohydrate sources. Pentoses such as xylose or arabinose, likewise, are not metabolized. Sucrose and maltose may be employed in place of glucose if supplied in relatively pure form, but attempts to utilize cane molasses, beet molasses, or hydrol (molasses from corn sugar

manufacture) in this fermentation were unsuccessful although they supported abundant growth.

Inoculum development. The influence of the age and volume of inoculum was explored, and pertinent results on the former are shown in figure 2. Liquid cultures were prepared from agar slants on successive days to provide inocula varying in age from 24 to 120 hours. Each of these was then employed to seed media of the same composition at a rate of 1 per cent by volume. The results show that, though the age of the inoculum did not markedly influence the time required to initiate riboflavin synthesis, considerably greater production was obtained from inocula incubated for only 24 hours. The young vigorous cells seemingly induced a rapid and more prolonged period of synthesis, whereas cultures made from older preparations produced riboflavin at a relatively slow
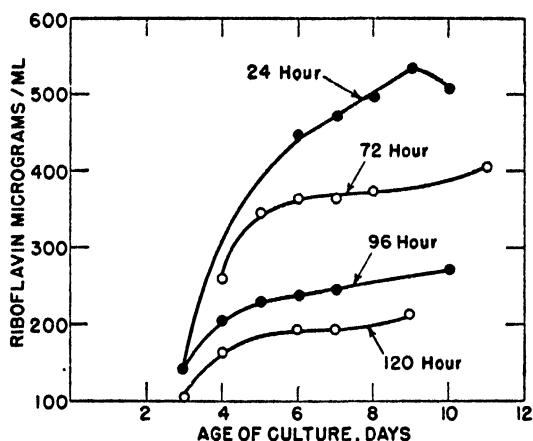
Figure 2. Influence of the age of *Ashbya gossypii* on the synthesis of riboflavin.

rate and for periods of short duration. A similar effect was observed with respect to the age of the slant culture from which liquid inocula were developed, transfers from young slants tending to be more productive. Because of the critical nature of this step, the practice of making daily transfers of both liquid and agar cultures was adopted in all subsequent experiments. In addition to the beneficial effect of employing young inocula, better riboflavin yields were generally secured when the volume of inoculum was equivalent to 0.5 to 1.0 per cent of the volume of medium. Levels appreciably above 1.0 per cent were less favorable, and a pronounced reduction in yield generally resulted with 10 per cent of inoculum.

Medium composition. In the light of the observations of Wickerham *et al.* (1946) it must be concluded that the failure of earlier workers to observe more than traces of riboflavin in cultures of *A. gossypii* was due largely to the media employed. Gorodkowa and Sabouraud media (Guilliermond *et al.*, 1935), and malt extract media (Stelling-Dekker, 1931) gave only slight quantities of riboflavin even over extended incubation periods. Likewise, *A. gossypii* grows readily without producing visible amounts of riboflavin in a medium composed of thiamine, biotin, inositol, glucose, asparagine, and potassium nitrate and other

inorganic salts (Robbins and Schmidt, 1939). On the other hand, Wickerham and co-workers noted that both agar and liquid media containing carbohydrate, peptone, and yeast extract led to substantial vitamin synthesis. They did observe differences in the effectiveness of various lots of medium ingredients, particularly the yeast extract. Our efforts were directed, therefore, to the development of uniformly good media from available crude proteinaceous materials.

Corn steep liquor was found to be a very satisfactory substitute for yeast extract, and since it is available in abundance at low cost, it was incorporated into most of the test media. Little effort was expended in exploring other substitutes for yeast extract; however, distillers' solubles showed promise as an alternative material.

Replacement of the commercial peptone was somewhat more difficult. Materials such as soybean meal, linseed meal, cottonseed meal, corn gluten, and wheat gluten were tested. These gave lower and generally more variable results and none produced yields consistently comparable to peptone plus corn steep liquor. Materials of animal origin were considerably more effective replacements than plant products. Among those tested and found to support good riboflavin production in combination with glucose and corn steep liquor were animal stick liquor,[5] tankage, and meat scraps. Frequently, even greater riboflavin yields were obtained by subjecting these supplements to digestion with proteolytic enzymes prior to incorporation into the medium. Papain and trypsin were about equally effective. Products such as liver tankage, liver meal, and blood meal gave lower riboflavin synthesis than stick liquor and were not substantially improved by proteolysis. The one sample of fish stick liquor tested was inactive.

Examples of the yields obtained with several of these supplements are shown in table 2. Two media, each composed of 4.0 per cent glucose and 0.5 per cent corn steep liquor but with 0.25 per cent peptone in the one case and 0.5 per cent peptone in the other, were used as standards of comparison. The yields from 50 trials with these standard media averaged 392 and 395 μg of riboflavin per ml, respectively. Although the variations between successive runs were considerable, the higher level of peptone generally gave superior results. By taking advantage of the preferred conditions, as was possible during the 10 latest experiments, yields were less variable and averaged 575 and 555 μg per ml, respectively. In these later trials, more significance should be attached to the increase in average yields than to the influence of peptone concentration, since during this period several different lots of supplements were evaluated. The improvement of yields by modifying the type and ratio of medium ingredients suggests also that further studies along this line might be fruitful. This is strongly indicated by the highest unconfirmed single yield of 1,050 μg per ml obtained in a shake flask culture

[5] Stick liquor is a by-product of wet rendering which is obtained by suspending packinghouse wastes in water and heating under pressure to release the fat. The fat and other insolubles are then separated and the liquid evaporated. Stick liquor is the condensed liquid phase of this digestion.

and 1,060 μg per ml and 1,420 μg per ml obtained in parallel 30-liter fermentations.

Sterilization of media. Prolonged autoclaving of media was observed to have a marked adverse effect on riboflavin production. In table 3 are presented results

TABLE 2

The suitability of various animal proteinaceous materials as substitutes for peptone in the production of riboflavin

FOUR PER CENT GLUCOSE PLUS	AGE OF CULTURE	
	8 days μg/ml	
0.5% CSL* + 0.25% peptone...	392†	
0.5% CSL + 0.50% peptone.......	395†	
	5 days	7 days
0.5% CSL + 0.25% beef scraps.....................................	186	296
0.5% CSL + 0.50% beef digester tankage...........................	270	356
0.5% CSL + 0.25% beef stick liquor...........................	178	304
0.5% CSL + 0.50% beef stick liquor...............................	164	312
0.5% CSL + 0.10% fish stick liquor...............................	37	38
0.5% CSL + 0.25% fish stick liquor...............................	13	14
Corn stillage + 0.25% peptone (dil. 1:1 H₂O).................	72	128
Corn stillage + 0.50% peptone.....................................	60	238
Corn stillage + 0.75% peptone.....................................	62	256

* Corn steep liquor.

† These are the average yields from 50 separate trials.

TABLE 3

Influence of sterilizing conditions on riboflavin yields

STERILIZATION TREATMENT	AGE OF CULTURE	
	8 days	10 days
	μg/ml	
Seitz filtration......................	678	676
Autoclave 15 minutes.......	648	700
Autoclave 30 minutes	680	700
Autoclave 45 minutes.......	494	586
Autoclave 60 minutes................................	308	346
Autoclave 75 minutes......	288	360
Autoclave 90 minutes.....	248	324

of a representative experiment to show the critical nature of this step. It may be noted that yields were sharply reduced when the autoclaving time exceeded 30 minutes at 121 C. After 90 minutes at this temperature, potencies were lowered from one-half to one-third those formed in the controls autoclaved for 15 minutes. This factor becomes of considerable importance when large volumes of medium are to be sterilized, as for example, in pilot-plant or commercial-scale operations,

and when the use of flash sterilizing techniques is indicated. The difficulty also was overcome to a considerable extent by sterilizing the medium ingredients separately.

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SUMMARY

Ashbya gossypii was found capable of synthesizing large amounts of riboflavin when it was propagated in certain media under conditions of submerged aerobic cultivation. Several factors were found to influence riboflavin production, the most important of which were concerned with the type and concentration of the medium constituents. Crude proteinaceous nitrogen sources were required, and combinations of corn steep liquor with certain materials of animal origin, e.g., animal stick liquor and tankage or meat scraps, were most satisfactory. These, with fermentable sugar, constituted a satisfactory commercial medium. Glucose, sucrose, or maltose served as adequate carbohydrate sources, but pentoses were not assimilated.

The use of small quantities of young inocula, a minimum sterilization time, and an efficient means of aeration were additional factors of importance.

The data indicate that a fermentation method for the commercial preparation of riboflavin either in concentrate or pure form might be based upon the use of *Ashbya gossypii*.

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THE ACTION OF STREPTOMYCIN

I. The Nature of the Reaction Inhibited

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Streptomycin, in contrast to penicillin, is known to affect the metabolic properties of "resting cell" suspensions (Benham, 1947; Bernheim and Fitzgerald, 1947; Fitzgerald and Bernheim, 1947; Geiger, 1947; Fitzgerald and Bernheim, 1948; Henry, Henry, Housewright, and Berkman, 1948; Wight and Burk, 1948). Although some of the results reported were doubtless due to secondary effects (for example, Fitzgerald and Bernheim, 1948), the effect of streptomycin upon amino acid oxidation following carbohydrate or organic acid oxidation, discovered by Geiger (1947), was traced, in an earlier report (Umbreit, 1949), to inhibition of the terminal respiration system possessed by *Escherichia coli*. Some evidence was provided (Umbreit, 1949) that the terminal respiration process in this bacterium involved a pyruvate-oxalacetate condensation and that streptomycin exerted its effect close to this reaction. In this paper experiments designed to test this hypothesis are described. The enzyme system carrying out the sensitive reaction has so far proved extremely refractory to the usual methods for isolation and separation. It has therefore been necessary to content ourselves with the type of information obtainable on resting cell suspensions. Because of the simultaneous occurrence of several competing reactions, such suspensions do not lend themselves to direct quantitative demonstrations of reaction mechanisms. The information obtainable by these methods is, however, sufficient to permit reasonable certainty and enables one to approach other problems connected with the action of antibiotics. Within the limitations of the resting cell technique we have been able to trace the mode of action of streptomycin somewhat further and have been able to apply this information to related problems, as demonstrated in subsequent papers of this series.

METHODS

The methods employed have been described previously (Umbreit, 1949). Oxalacetate was prepared from sodium diethyloxalacetate essentially as described by Umbreit *et al.* (1945) and recrystallized from acetone-benzene mixtures. Keto acids were determined by the method of Friedemann and Haugen (1943). All respiration studies were made at 37 C in 0.003 M phosphate buffer at pH 7. The organisms were grown for 16 to 18 hours at 37 C in 1-liter Erlenmeyer flasks containing 800 ml of medium consisting of 1 per cent each of Difco tryptone and yeast extract and 0.5 per cent K_2HPO_4 in distilled H_2O, harvested by centrifugation, washed, and suspended in distilled water to yield 0.5 mg bacterial nitrogen per ml. The age of the suspensions reported is the number of days such

suspensions were held at refrigerator temperatures. All data in this paper refer to the "Gratia" strain of *E. coli* (inhibited in growth by 9 μg streptomycin per ml) although the main features have been repeated on the "Murray" strain with essentially the same results. For clarity, all data have been corrected for a relatively low but somewhat variable endogenous respiration. Except when otherwise noted, the calcium chloride complex of streptomycin (Merck) was used at a level of 20 μg of the free base per ml.

EXPERIMENTAL RESULTS

Effect of streptomycin upon the oxidation of pyruvate, oxalacetate, and mixtures of these. On the assumption, as indicated by previous data (Umbreit, 1949), that streptomycin inhibits the oxalacetate-pyruvate condensation, the most direct approach would seem to be a study of the effect of streptomycin upon the
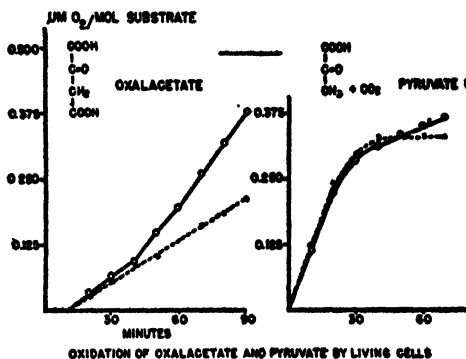
Figure 1. Comparative oxidation of oxalacetate and pyruvate by fresh cell suspension. One ml (= 0.5 mg bacterial N) *E. coli* (Gratia) suspension immediately after harvest in 0.008 M phosphate, pH 7.0. Twenty μM oxalacetate, 10 μM pyruvate, total volume 3 ml. Streptomycin (dotted line) at 20 μg free base per ml.

oxidation of oxalacetate and pyruvate in various combinations. When grown under the conditions described, the resting cell suspensions oxidize added pyruvate, apparently to acetate. The oxygen uptake per mole of added pyruvate is usually somewhat less than the theoretical quantity (0.5 O_2) required. With living cells there are usually some permeability effects as well as oxidative assimilation, so that the oxygen uptake on added pyruvate corresponds to 60 to 70 per cent of the theoretical value.

Fresh suspensions, less than 2 or 3 days old, possess an active oxalacetate decarboxylase, which rapidly converts added oxalacetate to pyruvate. As the cell ages (i.e., by storage of the suspension in the refrigerator), the oxalacetate decarboxylase enzyme becomes less and less active. Such aged cells retain the ability to oxidize pyruvate. It is therefore of importance to compare the activity on "old" and "young" suspensions, especially with respect to oxalacetate oxidation, since they actually possess a different enzyme pattern.

Data on the oxidation of oxalacetate and of pyruvate by fresh cell suspensions are given in figure 1. It is first evident that added oxalacetate is initially oxidized

at a slower rate than is pyruvate. Without streptomycin the rate of oxidation gradually increases; with streptomycin it remains constant. This type of data could be readily interpreted as follows: Oxalacetate (as indicated by its formula, figure 1) cannot be oxidized as such. Either it must be decarboxylated and the resulting pyruvate oxidized, or it may condense with pyruvate to enter a "citric acid cycle" (analogous if not identical to that occurring in the animal). In the latter case, the oxygen uptake would be due to oxidation of products successively formed during the cycle, after the initial condensation reaction. However, such a condensation requires that pyruvate be present, a condition that would obtain only after a time sufficient to enable adequate quantities of pyruvate to result from the decarboxylation of oxalacetate.

The increasing rate of oxidation, therefore, could be due to the occurrence of the oxalacetate-pyruvate condensation reaction, and the subsequent "Krebs (citric acid) cycle." If streptomycin inhibits this reaction, then the oxygen uptake can result only from the oxidation of both the added and newly formed pyruvate to acetate. Since the rate of oxidation of added pyruvate is considerably faster than the rate observed with oxalacetate, one may presume that the pyruvate formed from oxalacetate is oxidized to acetate when streptomycin prevents condensation and that the rate of oxidation is controlled by the rate of its formation from oxalacetate. Since the rate of oxygen uptake observed in the presence of streptomycin is constant, it would appear that the decarboxylation rate is constant. Similar considerations apply to the oxidation of pyruvate when no streptomycin effect is observed until the very late stages. One might presume that some carbon dioxide fixation occurs leading to small amounts of oxalacetate, which could condense with pyruvate to give continuing respiration only if streptomycin is absent and after the main body of pyruvate had been utilized.

It is, of course, recognized that the interpretation given above does not constitute proof that it is the oxalacetate-pyruvate condensation that is inhibited by streptomycin. The clearest proof would be the isolation of the enzyme causing this one step of the reaction and showing its inhibition by streptomycin. Since this has not yet been technically possible, it is necessary to employ an alternative approach. This consists of examining different types of systems in which the oxalacetate-pyruvate condensation would be evident in different manners and showing that streptomycin produces effects in these systems explainable by its inhibition of the condensation. It is probable that eventually alternative explanations can thus be eliminated, since possible alternatives for one system are not possible alternatives for other systems. So far three of these systems have been described: the oxidation of keto acids derived from amino acids (Umbreit, 1949), the oxidation of fumarate and pyruvate (Umbreit, 1949), and the example cited above, the comparison of oxalacetate and pyruvate oxidation by fresh cell suspensions that rapidly convert oxalacetate to pyruvate.

A fourth type of system is that of older cell suspensions in which oxalacetate is not rapidly converted into pyruvate. Data from such a cell suspension are given in figure 2. Here it will be noted that pyruvate is oxidized normally but oxalacetate is oxidized only very slowly, presumably because it cannot be decarboxy-

lated. If one then supplies the product of decarboxylation (i.e., if one supplies a mixture of oxalacetate and pyruvate), one should obtain the condensation reaction and this should be inhibited by streptomycin. This seems to be the case, in that oxygen uptake with both oxalacetate and pyruvate is considerably greater than with either alone, and the marked increase is inhibited by streptomycin. These results are thus compatible with streptomycin inhibition of the oxalacetate-pyruvate condensation.

The preceding example, however, has introduced a complication that requires further attention. It will be noted in figure 2 that a lag occurs before the mixed oxalacetate-pyruvate substrate is oxidized. Since this mixture contains pyruvate, why is not this substance oxidized as rapidly when added with oxalacetate as when added alone? After some study by keto acid analysis it became apparent that, when added alone or with oxalacetate, the pyruvate actually disappears from the reaction fluid very rapidly during this period; within the
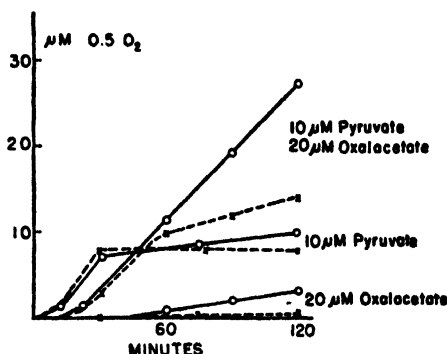
Figure 2. Oxidation of pyruvate and oxalacetate by older cell suspensions. One ml (= 0.5 mg bacterial N) *E. coli* (Gratia) suspension stored for 7 days in the refrigerator. Other conditions as in figure 1.

limits of the analytical methods when both are added, per mole of pyruvate lost a mole of oxalacetate disappears as well, with hardly measurable oxygen uptake. This initial disappearance of keto acids without comparable oxygen uptake was not significantly influenced by streptomycin. Data illustrating these points are given in figure 3. Here, during the lag phase of this reaction, at 15 minutes, 25 μM keto acid (out of 30 added) have been lost with an oxygen uptake of only 3 μM (0.5 O_2). It appears that during this early interval virtually all of the pyruvate (11 μM) has disappeared, and with it an equivalent amount of oxalacetate, without resulting in great oxygen uptake. An explanation for this effect may be found in the diagram of figure 4.

If it be presumed that the carrier system to which the hydrogen (from the conversion of pyruvate to acetate) is transferred can react with oxalacetate at a rate greater than can be accommodated by reaction A (to oxygen), then in the presence of both oxalacetate and pyruvate the oxalacetate can serve as a hydrogen acceptor and successfully compete with oxygen. Evidence in this direction is

furnished in table 1, in which hydrogen uptake by way of hydrogenase is meas-
ured. From these data it is apparent that a mixture of oxalacetate and pyruvate
takes up hydrogen rather rapidly, a finding that is evidence that they are rapidly

Figure 3. Oxygen uptake and keto acid loss. *E. coli* (Gratia), 0.5 mg N per ml, suspen-
sion stored for 3 days in the refrigerator. Other conditions as in figure 1.

Figure 4. Diagram illustrating competition between oxygen and oxalacetate.

TABLE 1

Hydrogen uptake by resting cells of E. coli

SUBSTRATE	Q_{H_2} (N)	
	− Streptomycin	+ Streptomycin
Pyruvate..............................	0	0
Fumarate........................	2,880	2,880
Oxalacetate............................	480	480
Oxalacetate + pyruvate...............	1,200	1,200

converted (at least partially) into malate and fumarate. Evidence for the exist-
ence of these reactions in this strain of *E. coli* and for the general validity of this
technique is given in an earlier publication (Lichstein and Umbreit, 1947).

It thus appears that the net result of adding a mixture of pyruvate and oxal-
acetate is to provide a mixture of acetate, malate, and fumarate. One would

expect, therefore, that malate (or fumarate) could be employed in the same manner as a mixture of oxalacetate and pyruvate to yield identical results without a lag period, providing the oxidation of pyruvate to acetate (or to "active acetate") were not an essential part of the process. The problem of "active acetate" will be considered in the next section. Malate, in fact, can be used in place of the oxalacetate-pyruvate mixtures without a discernible lag period in oxygen uptake and shows entirely comparable streptomycin effects. However, by both direct and indirect measurement the reactions outlined in figure 4 are not inhibited by
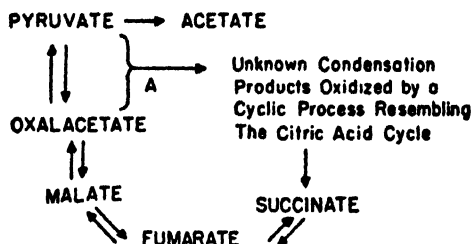
Figure 5. Diagram illustrating terminal respiration system in E. coli.

TABLE 2

Amounts of oxygen utilized on various substrates

SUBSTRATE	MOLES O₂/MOLE SUBSTRATE		THEORY TO ACETATE
	Normal	+ Streptomycin	
Pyruvate	0.44	0.42	0.5
Oxalacetate	>0.87	0.5	0.5
Malate	>2.3	0.77	1.0
Fumarate	1.42	1.0	1.0
Pyruvate + oxalacetate	>0.83	0.56	0.5

streptomycin. The streptomycin effects noted in figures 2 and 3 upon the addition of a mixture of oxalacetate and pyruvate must therefore reside in the inhibition of some other reaction than those given in figure 4. Presumably this is the condensation reaction.

A fifth type of system which would tend to implicate streptomycin inhibition of the oxalacetate-pyruvate condensation is the estimation of the oxygen utilized per mole of substrate supplied. The reasoning employed may be diagrammed as in figure 5. In the cells of E. coli grown in the manner described, pyruvate is oxidized to acetate. One would then expect that materials such as succinate, malate, etc., would, in the presence of streptomycin, also be oxidized to acetate. If streptomycin were absent, the condensation reaction (labeled A, figure 5) could occur leading to oxidation around the cycle (and eventually complete oxidation to CO_2 and H_2O). Therefore, if reaction A is inhibited by streptomycin, the oxygen uptake per mole of substrate (in the presence of streptomycin) should approach that required for oxidation to acetate. In the absence of streptomycin, the oxidation should be greater. Data on this point are given in table 2, from

which it is evident that in the presence of streptomycin oxygen uptake has proceeded largely to that required for oxidation to the acetate stage, whereas in its absence oxidation has proceeded much further. This is also especially evident in figure 3, in which 30 μM of keto acid (pyruvate and oxalacetate) were added (theoretical 0.5 O_2 uptake to acetate $= 30$); 28 μM (0.5 O_2) were taken up in the presence of streptomycin after which oxidation became slow, whereas without streptomycin 49 μM (0.5 O_2) were utilized in the same interval and the rapid oxidation continued. Therefore, in the absence of streptomycin, oxidation of a variety of substrates proceeds toward completion; in the presence of streptomycin, oxidation tends to proceed only as far as the acetate stage. As outlined, especially in figure 5, this further supports the conclusion that reaction A is the one acted upon by streptomycin.

The role of acetate or "active acetate." The tendency to oxidize substrate only to the acetate stage in the presence of streptomycin and the general accumulation of acetate in fermentations of inhibited strains, as reported by Henry *et al.* (1948, 1949), require consideration. This could come about in either of two ways: (1) If streptomycin prevented the oxalacetate-pyruvate condensation, substances that would normally be oxidized by this route form pyruvate, which is oxidized to acetate. (2) Alternatively, the oxidation of most substances proceeds through the "active acetate" stage and streptomycin interferes with the metabolism of "active acetate."

The latter possibility is especially pertinent since one of the proposed mechanisms of the "oxalacetate-pyruvate" condensation consists of a condensation of oxalacetate and "active acetate." Such a reaction has recently been demonstrated by Stern and Ochoa (1949). It is evident that with cells grown as described any effect must be upon "active acetate," since added acetate is oxidized only very slowly.

The term "active acetate" is, however, a misleading one inasmuch as several active 2-carbon intermediates may exist. As summarized by Gurin and Crandall (1948), the 2-carbon precursor of acetoacetate, the 2-carbon precursor of acetate, the 2-carbon precursor derived from pyruvate which condenses with oxalacetate, and the product of the reaction between acetate and adenosine triphosphate may be different substances. The problem that concerns us here is, Does the oxalacetate-pyruvate condensation go through a 2-carbon intermediate recognizable as acetate? By "direct condensation" of oxalacetate and pyruvate we do not necessarily exclude the formation of a transitory 2-carbon intermediate but mean only that, if it does exist, its origin is directly from pyruvate and that it does not exchange, to any great extent, with acetate.

Experimentally, the problem of distinguishing between these alternatives when one must employ living cell suspensions is not a simple one and the conclusions are always subject to the uncertainty that the living cell may follow pathways of metabolism of which we have no knowledge. However, within these limitations it can be demonstrated that the "direct" condensation of oxalacetate and pyruvate is the most likely alternative. Although many data have been collected on this point, it is pertinent to cite only two examples.

Acetate added to suspensions of *E. coli*, grown as described, is attacked only slowly. At times its oxidation is not affected by streptomycin; with some suspensions acetate oxidation is partially inhibited. The whole reaction is so slow, however, that its consideration may be postponed. The oxidation of pyruvate is not influenced by streptomycin except in the later stages (at which time streptomycin could have influence under either of the hypotheses outlined). The oxidation of acetate is not stimulated by oxalacetate; that of pyruvate is (as shown in figures 2 and 3). If the pyruvate were serving as a source of "active acetate" which would enter the citric acid cycle, it should be possible to oxidize pyruvate to "active acetate" whose further oxidation should be stimulated by oxalacetate and inhibited by streptomycin.

Data on this point are given in figure 6. In one series (A) 20 μM of oxalacetate were added at 75 minutes; the oxidation at 220 minutes amounted to 250 μl O_2 due to oxalacetate addition. In a second series (B) 10 μM of pyruvate were added
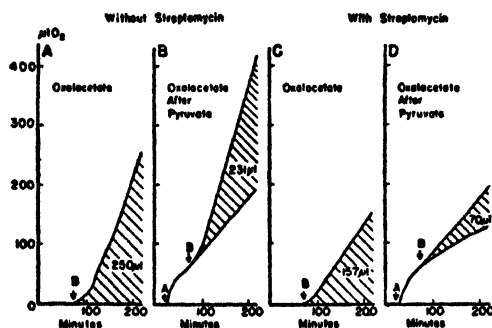
Figure 6. The effect of the previous oxidation of pyruvate upon the oxidation of oxalacetate. *E. coli* (Gratia), 0.5 mg N per ml, in 0.003 M phosphate. Suspension stored in the refrigerator for 5 days. At point A, 30 minutes, 10 μM of pyruvate added (B, D); at point B, 75 minutes, 20 μM of oxalacetate added.

at 30 minutes and had completed the most rapid oxidation period by 60 minutes. At 75 minutes 20 μM of oxalacetate were added, which, at 220 minutes, had resulted in 231 μl additional oxygen uptake due to the oxalacetate. It seems apparent that the oxalacetate has not stimulated the oxidation of "active acetate" derived from pyruvate. The comparable figures when malate was employed rather than oxalacetate were A = 524, B = 458. The comparable experiments in the presence of streptomycin show for oxalacetate alone (C) 157 μl, and for oxalacetate after pyruvate (D) 70 μl. It is possible that the presence of acetate ("active acetate") from pyruvate oxidation is inhibiting the oxidation of oxalacetate when all this must go through the pyruvate stage, as presumably is the case in the presence of streptomycin. The comparable figures for malate are: C = 307, D = 234. It appears that the presence of the end products of pyruvate oxidation (acetate and "active acetate"), if anything, slightly inhibits the oxidation of oxalacetate or malate. The oxidation of "active acetate," derived from the oxidation of pyruvate, is not stimulated by the addition of oxalacetate, with or without streptomycin.

Secondly, if cells of this strain of *E. coli* are grown on the identical medium but on a shaking machine in air, they possess the ability to oxidize added acetate. The mechanism for this oxidation is not known. The amounts of oxygen taken up per mole of acetate added vary over a wide range, and we have not been able to obtain a clear picture of the reaction mechanism. Oxalacetate, for example, does not stimulate the oxidation of acetate by these cells. However, the acetate oxidation system, whatever it may be, is entirely insensitive to streptomycin. Since one might presume that it involved "active acetate," the oxidation of "active acetate" would thus not be sensitive to streptomycin. As mentioned, the probable existence of more than one active 2-carbon compound does not permit the elimination of a 2-carbon compound from the sensitive reaction, but only permits one to conclude that it is not the further metabolism of acetate which is inhibited by streptomycin.

We have therefore concluded that we are dealing with an "oxalacetate-pyruvate" condensation rather than with the metabolism of "active acetate." Admittedly this cannot be completely eliminated by these experiments. The situation is that all efforts to implicate "active acetate" have failed.

The fact that a system for oxidizing acetate can be made evident in these cells by growth in air requires a certain precaution in the growth of cells for streptomycin studies. If too much air is available during growth so that the oxidation of pyruvate does not stop at the acetate stage, the streptomycin effects on the oxidation of oxalacetate become less evident because acetate oxidation contributes to the oxygen uptake of the streptomycin-inhibited culture. In this sense the cultural conditions are important for demonstrating the streptomycin inhibition, but we believe this to be due to the necessity of minimizing alternative routes of metabolism so that the sensitive reaction is made evident.

Penetration. The considerations of the previous sections tend to support the supposition that streptomycin acts by inhibiting the oxalacetate-pyruvate condensation. There was, however, one type of evidence reported previously (Umbreit, 1949) which is not in accord with this concept. It was found that streptomycin exerted its effect on threonine oxidation only when it was present during the previous oxidation of fumarate and did not have any great effect when it was added with the threonine. This would suggest that streptomycin was acting in some manner other than inhibiting the oxalacetate-pyruvate condensation. Upon further study, however, it appeared that there was a time interval involved before streptomycin exerted its effect and that, in the previously reported experiments, most of the threonine oxidation had been completed before streptomycin had had time to act.

Data pertaining directly to the oxalacetate-pyruvate condensation are given in figure 7. Here the cells were allowed to respire without added substrate for 60 minutes. Streptomycin was added to one flask at zero time and to a second flask at 60 minutes; none was added to the third flask. In all cases oxalacetate was tipped in at 60 minutes, having incubated in the side arm of the flask over this period. Since this substance is decarboxylated spontaneously, in effect one was adding a mixture of pyruvate and oxalacetate with the latter predominating. It is obvious that streptomycin has much less effect when added with the oxal-

acetate (at 60 minutes) than when added an hour earlier. Figure 7 (second part) shows the rate of oxidation subsequent to the oxalacetate-pyruvate addition when streptomycin was added at intervals before and after the addition of oxalacetate (added at A). The results indicate that, under the conditions of the test, about 30 minutes' contact between streptomycin and the cell suspension is required before streptomycin is fully effective in preventing the condensation. The time interval required to achieve apparently complete inhibition varies with the cell suspension, the salt concentration, and the level of streptomycin, but no detailed study of the exact influence of these factors has been made.

Three apparent hypotheses could account for this time interval. These are: (1) Time is required to permit the streptomycin to diffuse into the cell and to reach inhibitory concentrations inside the cell (which may be much less than the concentration of streptomycin externally). (2) A time interval is required to
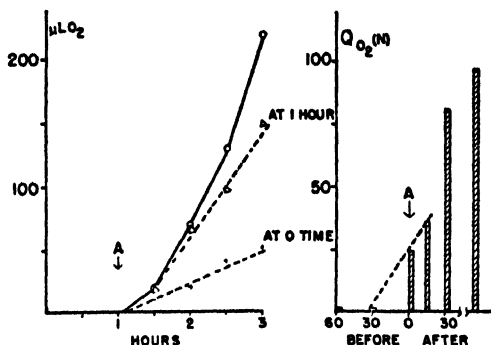
Figure 7. The effect of time of addition of streptomycin upon the oxidation of oxalacetate. See text.

permit the streptomycin to react with the enzyme. (3) Streptomycin is transformed by the metabolism of *E. coli* into a toxic agent and time is required for this transformation.

We have so far been unable experimentally to distinguish between these alternatives in *E. coli*. Since, however, the time lag in the animal preparations seems to be largely a matter of permeability, we have assumed that in the case of *E. coli* the first hypothesis was probably correct.

It is of some interest to examine the reversibility of the streptomycin effect. Cells of susceptible *E. coli* were therefore exposed to streptomycin for a period of 30 to 120 minutes. After 30 minutes' exposure the oxalacetate-pyruvate condensation was markedly inhibited (table 3). After exposure the cells were centrifuged, the supernatant was removed, and the cells were suspended in water for varying periods of time, up to 18 hours—but no relief from streptomycin inhibition was obtained. From these experiments it would appear that once streptomycin has reacted with the sensitive enzyme mere exposure to water does not relieve the inhibition.

It is obvious that these results differ somewhat from those of Berkman *et al.*

(1948) on the desorption of streptomycin, but it is possible that the actual amount of streptomycin required for inhibition is much lower than could be detected by assay techniques. It is further evident that no real explanation is available for the killing effect of streptomycin inasmuch as cells exposed to streptomycin under the conditions of our study are not rapidly killed and are apparently capable of continued growth when inoculated into a favorable medium.

Specificity of the reaction inhibited. Ample data have been presented to illustrate that the proposed site of action of streptomycin will account for the inhibitions observed with several substrates. Therefore the inhibitions observed are apparently due to inhibition of essentially one reaction in the systems studied. The next problem of specificity is whether the inhibition of this reaction correlates with the antibiotic activities of streptomycin derivatives. Without presentation of the details it can be reported that the reaction was inhibited by streptomycin (as the CaCl$_2$ complex, the hydrochloride, the sulfate, etc.), by dihydrostreptomycin, and by mannosido streptomycin (streptomycin B) at amounts

TABLE 3

Reversibility of streptomycin inhibition

CELLS NOT EXPOSED TO STREPTOMYCIN			CELLS EXPOSED TO STREPTOMYCIN		
First exposure time (Water)	Second exposure time (Water)	Q_{O_2} (N)	First exposure time (Streptomycin)	Second exposure time (Water)	Q_{O_2} (N)
min	*min*		*min*	*min*	
30	0	404	30	0	122
120	30	438	120	30	120
120	18 (hr)	324	120	18 (hr)	88

comparable to those required for inhibiting the growth of the organism. Streptomycin derivatives that showed no antibiotic effect did not inhibit the reaction at 500 μg per ml or above. Among the compounds tested were the following: streptidine, streptamine, strepturea, streptomycin oxime, streptomycin hydroxylamine, dideguanyl dihydrostreptomycin, dipyridino dihydrostreptomycin, and phenyl-pentacetyl dihydrostreptomycin. It therefore appears that the same specificity is associated with this reaction that is associated with the inhibition of growth.

Further observations. The indirect approach that was necessarily employed and our lack of information on the exact nature of the reaction inhibited required that certain other studies be made. Since these are largely negative they are described only briefly here. Upon the assumption that a direct condensation between pyruvate and oxalacetate was occurring, efforts were made to detect the presumed 7-carbon intermediate. The alleged 7-carbon intermediate might be a ketonic tricarboxy acid. Efforts were therefore made to prepare a 2,4-dinitrophenylhydrazone. The reaction was stopped at intervals during the first 30 minutes by acid 2,4-dinitrophenylhydrazine and the resulting mixture separated by means of paper chromatographs. Although oxalacetate, pyruvate, and un-

reacted reagent could be readily detected, we were able to find no other spot on the papers developed in various solvent mixtures which could be attributed to an unidentified intermediate.

Studies were also made in an effort to locate a "6-carbon" intermediate on the assumption that this would resemble citric or cis-aconitic acid in its chemical reactions. A rather nonspecific method for citric acid was employed (Saffran and Denstedt, 1948) in the hope that it would also react with the analogous "6-carbon" intermediate, but aside from some interference by residual oxalacetate in the early stages, no positive evidence for a citric-acid-like intermediate was found. It seems possible that the intermediates are rapidly transformed and that they thus escape detection. Streptomycin does not lead to the accumulation of a "7-carbon" or a "6-carbon" intermediate by any of the tests we have been able to apply.

The presence of a "citric acid cycle" in E. coli has always been a matter of some doubt since methods applicable to animal tissues do not work with E. coli. The addition of citric, cis-aconitic, and α-keto-glutaric acid salts to suspensions of E. coli results in only a slight oxidation. Glutamate, however, is very rapidly oxidized, and in the early stages a keto acid, with the properties of α-keto-glutarate, accumulates and later is rapidly removed. We have therefore assumed that α-keto-glutarate (as well as perhaps citrate and cis-aconitate) are slowly oxidized because they do not readily penetrate the intact cell.

It has been reported that lipositol is an antagonist of streptomycin (Rhymer et al., 1947). Lipositol, prepared from soybean lecithin by the method of Woolley (1943), did not antagonize inhibition by streptomycin of the oxalacetate-pyruvate oxidation. Neither was phytate or inositol antagonistic. These materials also did not prevent streptomycin inhibition when they were present for some time previous to the addition of streptomycin.

SUMMARY

Streptomycin specifically inhibits an oxidative reaction in susceptible strains of Escherichia coli. This reaction is apparently the "oxalacetate-pyruvate" condensation and when inhibited prevents a variety of substances from entering the terminal respiration system that resembles the citric acid cycle.

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THE ACTION OF STREPTOMYCIN

II. The Metabolic Properties of Resistant and Dependent Strains

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The studes reported in previous papers (Umbreit, 1949; Oginsky, Smith, and Umbreit, 1949) provide an experimental approach to some of the problems of resistance to streptomycin and dependency upon streptomycin. This paper is concerned with the examination of the metabolic reactions of strains of *Escherichia coli* that have become resistant to or dependent upon streptomycin. The data reported here refer to strains that have been cultivated for some time under the conditions described and that are stabilized in the sense that they have had an adequate chance to become thoroughly adapted to the streptomycin-containing environment.

METHODS

Manometric methods, substrate preparations, and cell growth and suspension procedures were the same as those described previously (Oginsky, Smith, and Umbreit, 1949). The sensitive and dependent strains of the "Murray" *E. coli* were obtained from the Department of Microbiology of the New Jersey Agricultural Experiment Station. The resistant variants of both the "Murray" and "Gratia" strains were developed by Mr. Joseph Pietrowski. The dependent variant of the "Gratia" strain was isolated in our laboratory by growing the streptomycin-sensitive parent culture in 80 μg per ml of streptomycin. Limiting streptomycin concentrations permitting growth of the resistant organisms in Difco brain heart medium were 35,000 μg per ml for the "Murray" strain and 20,000 μg per ml for the "Gratia." The lowest streptomycin concentration permitting growth of the dependent organisms in a synthetic medium containing glucose, ammonium sulfate, K_2HPO_4, and inorganic salts was slightly above 10 μg per ml for both strains.

RESISTANT VARIANTS

Observations have shown that the parent strain and the derived resistant variants differ metabolically in three ways. The sensitive parent strains are able to benefit by the presence of air during growth, are able to oxidize rapidly the intermediates of the citric acid cycle, and possess an enzyme causing the oxalacetate-pyruvate condensation. The resistant variants do not have these properties. Data supporting these conclusions follow.

Growth. The data of table 1 record the growth of strains of *E. coli*, after 48 hours at 37 C, in a yeast extract tryptone medium to which were added increasing quantities of glucose under two conditions. The first part of the table, labeled "stationary cultures," represents growth in flasks well-filled with medium (800

ml per liter flask) in which the circulation of air is limited. Little difference was evident between the growth of the resistant and the sensitive forms. In our hands, the resistant strain has tended to grow rather poorly on continued cultivation in this medium over a period of months. The second portion of the table, labeled "shaken cultures," represents growth in the same medium for the same length of time on a shaking machine (100 ml medium per 500-ml flask) to provide vigorous aeration. Under these conditions the sensitive strain responds to the availability of air by a roughly 10-fold increase in growth; the resistant variant responds little, if at all. The results are essentially the same whether or not streptomycin is present in the medium on which the resistant variant is

TABLE 1

The growth of sensitive and resistant strains in complex media

PER CENT GLUCOSE	"GRATIA" STRAIN		"MURRAY" STRAIN	
	Sensitive	Resistant	Sensitive	Resistant
	Stationary cultures			
0.0	16*	8	10	12
0.1	28	16	25	24
0.5	30	20	40	32
1.0	30	20	40	30
	Shaken cultures			
0.0	284	8	264	12
0.1	320	16	284	24
0.5	332	28	360	32
1.0	250	24	250	68

Base medium: 1 per cent tryptone, 1 per cent yeast extract, and 0.5 per cent K_2HPO_4.
* Growth expressed in terms of μg bacterial nitrogen per ml.

grown. So far we have not observed reversion of the resistant to the sensitive form.

Oxidation. The data of table 2 record the ability of resistant and sensitive strains to oxidize the components of the citric acid cycle. It is apparent that the oxidative abilities of the resistant variant are considerably less on these substrates than are those of the sensitive strain. In the case of the resistant variants, the rates of oxidation of substances such as succinate or fumarate are somewhat variable, depending upon the conditions of growth, but never does oxidation of these substances approach that by the sensitive strain.

Oxalacetate-pyruvate condensation. Data on the oxidation of pyruvate, oxalacetate, and mixtures of the two are given for the "Gratia" sensitive and resistant pair in figure 1. In the sensitive strain, the typical reactions described in the previous paper (Oginsky, Smith, and Umbreit, 1949) are observed. In the resistant variant, however, it is evident that oxalacetate and pyruvate are oxidized

no faster than oxalacetate alone, and, therefore, the oxalacetate-pyruvate condensation is apparently absent. This phenomenon of loss of the oxalacetate-pyruvate condensation is not confined to *E. coli*. A culture of *Pseudomonas*

TABLE 2

Oxidation rates on substrates related to the citric acid cycle by sensitive and resistant variants of E. coli

SUBSTRATE	"GRATIA" STRAIN		"MURRAY" STRAIN	
	Sensitive	Resistant	Sensitive	Resistant
	Q_{O_2} (N)	Q_{O_2} (N)	Q_{O_2} (N)	Q_{O_2} (N)
Lactate	680	120	1,020	230
Pyruvate	480	120	540	152
Oxalacetate	80	60	50	40
Oxalacetate plus pyruvate	400	80	400	160
Malate	260	80	472	80
Fumarate	360	80	540	88
Succinate	520	120	680	122
α-Ketoglutarate	60	0	60*	0
Citrate	94	12	56	—
cis-Aconitate	153	10	—	—

* Glutamate is oxidized at Q_{O_2} (N) = 440.
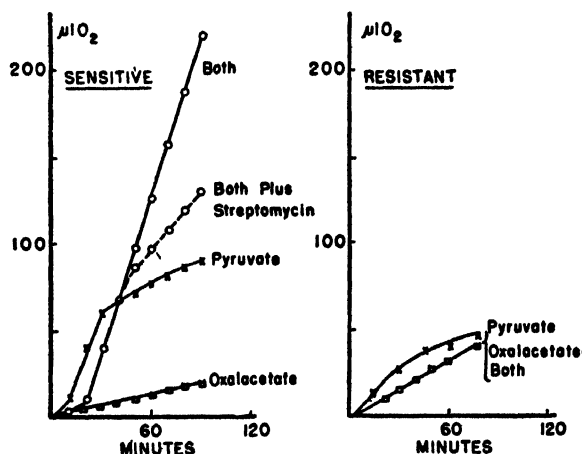
Figure 1. Oxidation of oxalacetate and pyruvate by sensitive and resistant strains. In all cases, 10 μM pyruvate and 20 μM oxalacetate were added where indicated.

aeruginosa, made resistant to streptomycin, lost the oxalacetate-pyruvate condensation reaction present in the sensitive parent strain.

DEPENDENT VARIANTS

Observations comparable to those made on the resistant cells have shown that there is much less difference in the metabolic pattern between the sensitive and

dependent cells than between the sensitive and resistant foms. Both sensitive and dependent cells respond to the presence of air by increased growth, and some of the dependent variants oxidize the intermediates of the citric acid cycle; others do not. The primary point of metabolic difference is that the sensitive

TABLE 3

Growth of the sensitive and dependent "Murray" strains

PER CENT GLUCOSE	SENSITIVE	DEPENDENT
	Stationary cultures	
0.0	10*	12
0.1	25	20
0.5	40	32
1.0	40	36
	Shaken cultures	
0.0	264	220
0.1	284	280
0.5	360	212
1.0	250	212

* Conditions as in table 1. Growth expressed as μg bacterial nitrogen per ml.

TABLE 4

Oxidation rates on substrates related to the citric acid cycle by sensitive and dependent variants of E. coli

SUBSTRATE	"GRATIA" STRAIN		"MURRAY" STRAIN	
	Sensitive	Dependent	Sensitive	Dependent
	Q_{O_2} (N)	Q_{O_2} (N)	Q_{O_2} (N)	Q_{O_2} (N)
Lactate....................................	680	660	1,020	420
Pyruvate................................	480	480	540	480
Oxalacetate.............................	80	160	50	200
Oxalacetate plus pyruvate............	400	760	400	400
Malate....................................	260	—	472	—
Fumarate................................	360	460	540	60
Succinate...............................	520	760	680	120
α-Ketoglutarate.......................	60	120	60	30
Citrate....................................	94	—	56	—
cis-Aconitate...........................	153	75	—	—

strains póssess the oxalacetate-pyruvate reaction, whereas the dependent variants do not. Data supporting these conclusions follow.

Growth. It is evident from the data of table 3 that the dependent variant responds to aeration by an approximately 10-fold increase in growth. There seems to be little difference between the sensitive and dependent strains in this respect. Data for the "Murray" strains only are given in the table, but those for the "Gratia" strains are entirely comparable.

Oxidation. It is apparent from table 4 that the two dependent strains differ.

The dependent "Gratia" variant possesses the ability to oxidize the citric acid cycle intermediates, sometimes faster than the sensitive parent does. In the dependent "Murray" variant this property appears to have been considerably reduced.

Oxalacetate-pyruvate condensation. Data comparing the properties of the sensitive and dependent variants of the "Gratia" strain are given in figure 2. At first inspection the oxidation of the compounds listed appears to be essentially similar in the two cases. If, however, one compares the rate of oxalacetate oxidation in the sensitive and dependent strains, it will be noted that oxalacetate itself is oxidized much more rapidly by the latter. Apparently the rate of decarboxylation of this compound to pyruvate in the dependent strain is much
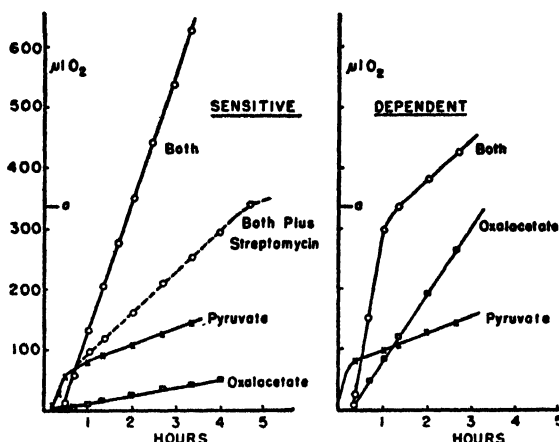
Figure 2. Oxidation of oxalacetate and pyruvate by sensitive and dependent strains. Conditions as in figure 1. Indentical data are obtained for the dependent strain in the presence or absence of streptomycin.

faster than in the sensitive. With the dependent variant the total oxygen uptake observed after rapid oxidation of the mixture of oxalacetate and pyruvate ceases is 290 μl O_2. The substrate for the oxalacetate-pyruvate system consisted of 10 μM of pyruvate and 20 μM of oxalacetate, i.e., 30 $\times$ 22.4 μl = 672 μl of substrate added. The oxygen uptake observed is thus $\frac{290}{672}$ = 0.43 O_2 per mole of substrate, i.e., approximately the amount (0.5 O_2, marked as point *a* on both graphs) required to oxidize both components to acetate. In the case of the sensitive strain the whole reaction system is slower. The data on the sensitive strain show that the oxidation of this substrate mixture in the absence of streptomycin proceeds farther than can be accounted for by oxidation to the state of acetate. By 180 minutes the sensitive strain has taken up 500 μl O_2 in the absence of streptomycin and the rate of oxidation is continuing undiminished, but with streptomycin only 230 μl O_2 has been utilized over the same period.

We interpret these results to indicate that in the sensitive cell the oxalacetate-pyruvate condensation occurs; in the dependent cell the oxalacetate is decar-

boxylated to pyruvate and the latter oxidized to acetate, without a condensation occurring between these materials.

DISCUSSION

There would appear to be, a priori, at least four principal methods by which resistance to streptomycin or other drugs could be achieved. These are:

(1) A system to destroy the drug, similar to penicillinase, might develop.

(2) The resistant cells might become impermeable to the antibiotic, as, for example, the penicillin resistance of yeasts (Maass and Johnson, 1949).

(3) The resistant cells might form a resistant enzyme, as has been reported for sulfonamide resistance (Gots and Sevag, 1948).

(4) The sensitive reaction might by "by-passed," i.e., an insensitive reaction accomplishing the same purpose might be substituted, so that the inhibited reaction might no longer be vital.

With the variants that we have studied, it appears that the last alternative does occur and that the oxalacetate-pyruvate condensation has been eliminated. This is not to imply that all strains resistant to streptomycin will have developed this alternative, but only that we have dealt with examples of this type.

Consideration of these results has suggested the following hypothesis. Since the resistant and dependent variants no longer possess a discernible oxalacetate-pyruvate condensation, they have developed an alternative pathway capable of forming substances, vital to the cell, which result from the condensation in the sensitive strain. The dependent variant has developed the ability to form such substances from streptomycin. Cited below are the types of experiments we have made in an unsuccessful attempt either to establish this hypothesis or to eliminate it completely.

One would presume that, if the hypotheses outlined above were correct, the resistant cell would possess these vital substances, and killed cells or extracts thereof might permit the sensitive strain to grow in the presence of streptomycin. Experimentally we have so far been unable to demonstrate this effect. If the resistant cell is able to "by-pass" the streptomycin-inhibited reaction, it ought to be possible for the sensitive cell to do so. For example, Green, Iverson, and Waksman (1948) report that pyruvate and fumarate antagonize the action of streptomycin to some extent, i.e., permit growth of the sensitive strain in the presence of streptomycin. However, in our own studies, when citric or cis-aconitic acid (both of which are oxidized relatively slowly by the resting cells) were supplied as the presumed products of the oxalacetate-pyruvate condensation, they did not permit growth of the sensitive strain in the presence of streptomycin. The same was true for various preparations of lipositol. We have not yet found a method for growing the sensitive strain in the presence of streptomycin (at levels inhibiting the growth of the parent strain) unless it is converted to a resistant or dependent form. One would certainly expect that the resistant variant, having lost such a large and important segment of its metabolism, would develop added nutritive requirements and would require compounds that the sensitive strain would normally synthesize. This appears to be the case for some

strains, since Iverson and Waksman (1948) have reported the development of a requirement for lysine with the development of streptomycin resistance by *E. coli*. However, our resistant "Gratia" strain will grow on a simple synthetic medium; hence it does possess the ability to synthesize its growth essentials from glucose and mineral salts.

Assuming that the dependent strain synthesized its vital substances from streptomycin, we attempted to identify these substances by searching for materials, other than streptomycin, which would serve for the growth of the dependent variants. In any medium or in the presence of any supplement tried thus far, we have obtained no growth save when streptomycin or dihydrostreptomycin was present. The media employed have ranged from synthetic media, yeast extract and peptones, to brain heart infusion. Supplements employed alone and in combinations were the derivatives of streptomycin listed in the previous paper (Oginsky, Smith, and Umbreit, 1949), killed cells and extracts from sensitive or resistant strains, liver extracts, vitamins, amino acids, inositol, lipositol preparations, etc. As others have reported (Iverson and Waksman, 1948; Rake, 1948), we have been unable to devise a medium not containing the intact streptomycin molecule which would support growth of the dependent strains.

SUMMARY

Resistant and dependent variants of the streptomycin-sensitive *Escherichia coli* do not possess the ability to effect the oxalacetate-pyruvate condensation in detectable amount. This is the reaction inhibited by streptomycin in the sensitive strains, and it appears that the ability to grow in the presence of streptomycin depends upon the development of unknown reactions permitting the cell to dispense with this condensation.

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THE ACTION OF STREPTOMYCIN

III. The Action of Streptomycin in Tissue Homogenates[1]

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The previous papers (Umbreit, 1949; Oginsky *et al.*, 1949) demonstrate that one of the actions of streptomycin is to inhibit the condensation between oxalacetate and pyruvate in susceptible strains of *Escherichia coli*. It is, however, well known that the oxalacetate-pyruvate condensation is an important reaction in the animal cell. Why, then, is streptomycin able to enter the animal body and kill or inhibit the susceptible bacteria therein without harm, or at least comparable harm, to the animal?

There are at least three possible explanations. First, it is possible that the oxalacetate-pyruvate condensation in the animal differs in some way from the same reaction in the bacteria. A very attractive concept is that in the animal the pyruvate is first converted to "active acetate," which is the material condensing with the oxalacetate. Indeed, such a system has recently been reported for animal tissue (Stern and Ochoa, 1949). One might suppose that the bacteria possess a "direct" condensation (Oginsky *et al.*, 1949) of pyruvate and oxalacetate not involving "active acetate." Second, it is possible that the reactions in the animal and the bacteria are essentially identical but that streptomycin does not penetrate to the site of this reaction in the animal cell, whereas it does so in the bacteria. Third, it is possible that streptomycin is itself converted into a toxic substance by the enzymes of the bacterial cells, but that the animal does not possess this enzyme system.

The importance of the problem of why streptomycin is able to enter the animal body without appreciable harm to the animal necessitated study of the effect of streptomycin in animal tissues or preparations therefrom. Although the methods employed are necessarily those of animal metabolic studies, the problem is one of most interest to the bacteriologist and is therefore recorded here. The terms "water homogenate" and "isotonic homogenate" are defined under methods, but it is helpful at this point to mention briefly the recent information on the enzymatic properties of mitochondria. When it was demonstrated (Bensley and Hoerr, 1934; Hoerr, 1943; Claude, 1942, 1944; Claude and Fullam, 1945; Hogeboom, Schneider, and Pallade, 1947, 1948) that it was possible to obtain morphologically intact mitochondrial fractions from animal tissues by the use of isotonic media for the disruption of the cell, the enzymatic properties of these fractions were studied (Hogeboom, Claude, and Hotchkiss, 1946; Schneider, 1946*a*,*b*; Kennedy and Lehninger, 1948; LePage and Schneider, 1948; Schneider, 1948; Schneider, Claude, and Hogeboom, 1948). It has become apparent that

the "citric acid cycle" component enzymes and carriers are associated with the mitochondria. A more important consideration is that the mitochondria behave differently when carefully prepared in isotonic media than when "roughly" treated, as in water homogenates, and do not require supplementation with diffusible cofactors. The enzymes in or on these carefully prepared particles appear to be more accessible to certain metabolites than they are in the tissue (Cohen and Hayano, 1946), but they possess properties that sometimes differ greatly from the comparable enzymes isolated in the cell-free state. The reason for these differences, which most workers agree do exist, is a matter of present study.

However, to distinguish among the several possible mechanisms of streptomycin action cited above, it is necessary to demonstrate that one of two phenomena occurs: first, that the enzyme causing the reaction in the animal is not susceptible to streptomycin even in the cell-free state (thus showing that the enzyme systems are different, hypothesis 1 or 3), or, secondly, that it is susceptible to streptomycin providing one permits the streptomycin to penetrate to the centers of this reaction in the animal cell (hypothesis 2).

In tissue slices or isotonic homogenates of rat liver or rat kidney very high concentrations of streptomycin, of the order of 200 to 300 μg per ml, frequently have no effect. At times a level of 300 μg per ml will show some inhibition in homogenates. Lower levels of streptomycin show no effect. However, studies on the distribution and rates of excretion of streptomycin in the animal body (Boxer, Edison, and Hawkins, 1949) have led to the conclusion that little if any of the streptomycin penetrates the cell, most of it being, indeed, distributed in the extracellular body fluids. One is therefore inclined to begin with the hypothesis that streptomycin does not penetrate to the enzyme catalyzing the oxalacetate-pyruvate condensation.

Since in both isotonic and water homogenates the cell structure is destroyed and one has a suspension of cell-free particles (nuclei, mitochondria, and submicroscopic particles with few if any intact cells), one might presume that any permeability barrier to streptomycin would be eliminated. However, this is not necessarily the case, and the fact that in order to preserve the mitochondria one must employ isotonic media and that such isotonic preparations do not usually require supplementation with diffusible factors such as DPN or cytochrome c indicates that a "permeability" barrier still exists at the surface of the mitochondria.[2] The problem that concerned us was whether or not this barrier pre-

[2] An alternative hypothesis is currently being developed by Green and co-workers (Green, Loomis, and Auerbach, 1948; Cross, Taggart, Covo, and Green, 1949) under the name "cyclophorase." It is our understanding that the "cyclophorase" concept conceives of the differences between mitochondrial preparations and the individual enzymes after isolation as being due, not to a permeability barrier at the mitochondrial surface, but to the existence of these enzymes in a different state. One conceives of a very large protein aggregate with the enzymes and coenzymes locked into it in some degree of order. Such a mechanism would account for the phenomenon we now speak of as permeability effects. Homogenization in water, for example, could be thought of as either disrupting certain portions of this aggregate or as interfering with the permeability. Agents that alter these phenomena may be spoken of as altering permeability or as, in Green's terminology, "transforming agents." Experimental evidence as to which of these two views is the explanation

vents the streptomycin from reaching the oxalacetate-pyruvate condensation enzyme. This enzyme has been shown to be concentrated in the mitochondria (Schneider and Potter, 1949).

METHODS

Homogenates were made in an all-glass homogenizer, as described by Potter (1945), and the techniques and reagents described by Potter, LePage, and Klug (1948) and Potter, Pardee, and Lyle (1948) for determining oxalacetate oxidation were followed. The components used in the complete system were glass-distilled water to make 3.0 ml final volume, 0.4 ml of 0.5 M KCl, 0.1 ml of 0.1 M MgCl$_2$, 0.1 ml of 0.1 M K phosphate (pH 7.4), 0.1 ml of 0.01 M KATP (or K adenylate), and 0.5 ml of isotonic KCl (if water homogenates were used). If less than 0.5 ml of isotonic homogenates were used, sufficient isotonic KCl was added to be equivalent to 0.5 ml. As oxidation substrates, 0.2 ml of 0.1 M pyruvate, 0.2 ml of 0.1 M oxalacetate, or an equivalent amount of 0.1 M fumarate was employed. The oxalacetate was prepared just before use. ATP was purified from commercial products by the methods described by LePage (1945). There was little difference between supplementation with ATP or adenylate; in most cases the latter was employed. Since it has been shown (Pardee and Potter, 1948) that fumarate will not be oxidized in this system unless the oxalacetate formed is removed, fumarate was frequently substituted for oxalacetate, particularly when long incubation periods were employed.

Two types of homogenates were used. Each was prepared with 9 ml of fluid per gram of fresh tissue. The "water homogenates" were prepared with ice-cold glass-distilled water; the "isotonic homogenates" were prepared with either 1.15 per cent KCl (to which had been added 0.3 ml of 0.1 M KHCO$_3$ per 100 ml to give a pH of 7.5) or with 8.5 per cent sucrose. We found very little difference between 0.9, 1.15, and 1.23 per cent KCl, or 8.5 per cent sucrose; hence for convenience only the data with 1.15 per cent KCl in kidney homogenates is reported. Liver and brain tissues were examined with entirely comparable results. We found little effect following the supplementation of isotonic homogenates further with cytochrome c and DPN; hence such supplements were used only when water homogenates were employed. The amounts used were 0.4 ml of cytochrome c (5 mg per ml) and 0.1 ml K DPN (coenzyme I, diphosphopyridine nucleotide) equivalent to 330 μg of DPN. We are indebted to Dr. G. A. Emerson for the animals, to Dr. J. E. Hawkins for specially treated animals, to Dr. G. A. LePage, University of Wisconsin, for a sample of purified ATP, and to Drs. LePage and V. R. Potter, University of Wisconsin, for very helpful advice. Streptomycin or dihydrostreptomycin was always employed in the form of the hydrochloride.

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for the observed phenomena is not sufficiently developed to render possible a valid decision between them at this time. However, rather than cite the two indistinguishable alternatives we have expressed the explanations and experiments in this paper in terms of the permeability hypothesis, recognizing that the "barrier" we speak of may be a chemical dislocation rather than a distinct membrane.

EXPERIMENTAL RESULTS

The problem of why streptomycin can enter the animal body without marked toxicity to the animal was approached through two types of experiments. The first approach consisted of comparing the activity of streptomycin in isotonic homogenates and in water homogenates, and was based upon the presumption that in the water homogenate the permeability properties of the mitochondria were impaired.

Figure 1 shows data on kidney homogenates comparing the activity on a mixed oxalacetate and pyruvate substrate in the presence or absence of 120 μg per ml of streptomycin. Dotted lines represent streptomycin treatment. To attain comparable conditions both systems carried out the oxidation in isotonic KCl supplemented with cytochrome c and DPN in addition to the usual supplements of ATP and magnesium. The data given are for unwashed homogenates
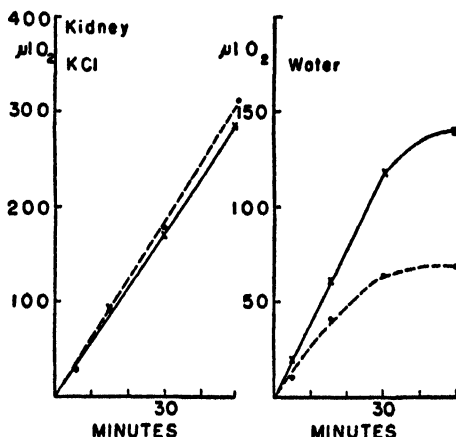
Figure 1. Comparative action of streptomycin on isotonic and water homogenates from rat kidney. Streptomycin (dotted lines) at a level of 120 μg (free base) per ml. Substrate, oxalacetate and pyruvate. Supplemented with cytochrome c and DPN. Thirty mg wet weight of tissue per cup.

used at a level of 30 mg wet weight of tissue per Warburg cup. At this dilution the addition of pyruvate alone results in very little oxygen uptake. In the isotonic KCl homogenates the activity is relatively constant for more than an hour; in the water homogenates it drops off rapidly after 30 to 40 minutes. From these data it is apparent that streptomycin inhibits the reaction in the water homogenates. However, relatively high levels of streptomycin are still required, concentrations in the range of 100 μg per ml being necessary. Concentrations lower than this have no effect.

The second approach to the problem of whether or not there exists a barrier to streptomycin at the surface of the mitochondria consisted of attempts to inhibit the reaction in isotonic homogenates by lowered concentrations of streptomycin under conditions designed to overcome or minimize any permeability barrier. Two types of experiments were employed. Data from the first

type are illustrated in figure 2. A kidney KCl homogenate was added to the cold reaction flasks and oxygen uptake was determined immediately after preparation. The data (curves A, figure 2) show no inhibition at a level of 60 μg of streptomycin per ml. A portion of this homogenate was held for 2 hours in the refrigerator. Oxygen uptake was then determined as before. The data (curves B, figure 2) show no inhibition by streptomycin. A third portion was added to the cold reaction flasks complete with substrate (and in one case streptomycin) and held for 2 hours in the refrigerator; oxygen uptake was then determined (curves C, figure 2). A fourth portion was treated in the same manner except that the substrates were added just before the end of the 2-hour refrigeration period (curves D, figure 2). When streptomycin has been in contact with the mitochondria for a period of 2 hours in the cold, an inhibition is evident. No
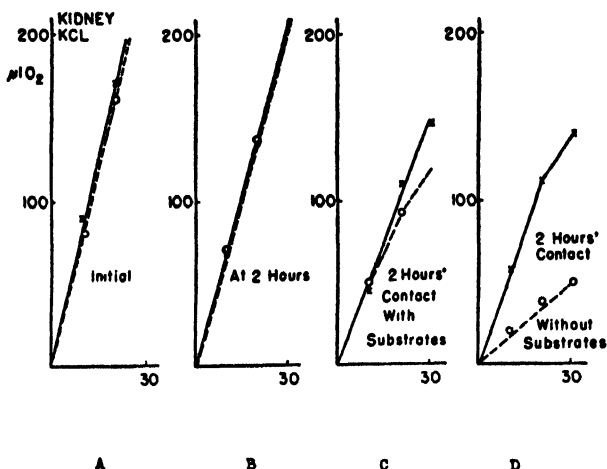
Figure 2. Influence of time of contact on the effect of streptomycin in isotonic rat kidney homogenates. Streptomycin (dotted lines) at a level of 60 μg (free base) per ml. Substrate, fumarate and pyruvate. Thirty mg wet weight tissue per cup. No additions of cytochrome c or DPN. See text for details.

supplementation with cytochrome c or DPN was used. Fumarate, which acts the same as oxalacetate, was employed here in order to circumvent the spontaneous decomposition of oxalacetate during the 2-hour refrigeration period. Two factors, however, have been varied in this experiment. In those cases in which streptomycin showed inhibition, not only was streptomycin present but the homogenate was diluted 1 to 6. This probably does not invalidate the results since the comparable flasks without streptomycin were also diluted to the same extent, and no great loss of activity is evident. However, experiments were designed to alter only one variable, that of streptomycin concentration. An isotonic homogenate was prepared and 0.6 ml (= 60 mg wet weight of tissue) were added to each of 7 cold empty Warburg flasks. Two of these were supplied with the reaction mixture, consisting of buffer, isotonic KCl, Mg^{++}, adenylic acid, oxalacetate, and pyruvate, and used immediately (one flask also received 100 μg strepto-

mycin). The remaining 5 received 0.1 ml of $MgCl_2$ and 0.3 ml isotonic KCl or dilutions of streptomycin in isotonic KCl so that the homogenate (thus diluted to 1.0 ml) would contain 0, 10, 25, 50, and 100 μg streptomycin per ml, respectively. This series was held in the refrigerator for 2 hours, the reaction substrates and supplements (less Mg, volume = 2 ml) were added and the oxygen uptake was measured. In this case the final concentration of streptomycin per flask at the time that oxidation occurred was only one-third that of the streptomycin recorded. Thus the homogenate exposed to 100 μg per ml of streptomycin oxidized the oxalacetate at a concentration of only 33 μg per ml. The concentration of streptomycin to which the mitochondria were exposed, however, is used in plotting the data shown in figure 3. Here it is evident that detectable inhibition is observed at as low at 10 μg per ml, and increasing inhibitions are evident at higher concentrations.
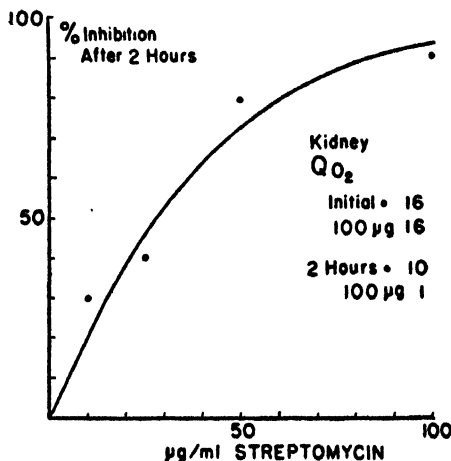
Figure 3. Degree of inhibition after 2-hour exposure to streptomycin. Details in text. Substrate, fumarate and pyruvate. Isotonic KCl homogenates.

DISCUSSION

It seems evident that the reaction carrying out the oxalacetate-pyruvate condensation in the animal *can* be inhibited by streptomycin. In the intact animal and, indeed, in isotonic preparations from the animal the reaction is not inhibited unless and until certain barriers, which resemble permeability barriers, are overcome. We have observed streptomycin inhibition at relatively low concentrations of streptomycin (50 to 60 μg per ml) in isotonic homogenates of liver, kidney, and brain whenever these homogenates were subjected to somewhat drastic conditions. Thus in "cyclophorase" preparations that were not adequately neutralized or in homogenates in which some heating occurred during homogenization, whenever activity remained it was more sensitive to streptomycin than the same activity in adequately treated preparations.

Two further points are of interest. In studies on penetration such as those of

figure 3, dihydrostreptomycin showed much less inhibition than streptomycin. It therefore appeared that it may have "penetrated" more slowly.

It was consequently of interest to determine whether, in the tissue of animals showing the effects of chronic toxicity of streptomycin or in those from animals acutely poisoned by streptomycin, the oxalacetate-pyruvate condensation was in any way impaired. Dr. J. E. Hawkins provided us with several animals of each type. Liver, kidney, and brain tissues of rats showing the vestibular and auditory symptoms of chronic streptomycin toxicity and those from rats that had been killed by intravenous or intercranial injections of streptomycin showed oxidation of oxalacetate-pyruvate at the same rate as controls.

SUMMARY

Streptomycin will inhibit the oxalacetate-pyruvate condensation in animal tissue as well as in the bacterial cell. In the intact animal permeability factors apparently prevent streptomycin from acting on the site of this reaction. These permeability barriers exist not only at the cell wall, but also at the mitochondria, and may be chemical as well as physical in nature. When the barriers are overcome, however, streptomycin does inhibit the oxalacetate-pyruvate condensation in animal tissue.

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THE INFLUENCE OF THE VITAMIN B COMPLEX ON THE GROWTH OF TORULOPSIS (CRYPTOCOCCUS) NEOFORMANS ON A SYNTHETIC MEDIUM

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The variation in morphology that occurs when fungi are grown on media of different composition is well known. In regard to the pathogenic fungi, such deviations and their general causes have been noted and commented upon by Fox and Blaxall (1896), Weidman and McMillan (1921), and Conant (1936). These observations have naturally led to a more thorough study of the growth requirements of the fungi in order to develop synthetic media that would minimize the variations.

Among the substances studied which have proved to be of importance in fungus nutrition are the vitamins, particularly those of the B complex. The need of *Trichophyton interdigitale* for at least four accessory substances, thiamine riboflavin, inositol, and pantothenic acid, for satisfactory growth was demonstrated by Mosher *et al.* (1936). A survey of the literature on vitamin requirements of the fungi by Robbins and Kavanagh (1942) indicated that, although few of the pathogenic fungi had been studied, growth of *Microsporum fulvum, Trichophyton crateriforme, Trichophyton rosaceum, Cryptococcus* (Busse-Buschke), and *Sporotrichum schenckii* in a synthetic medium was stimulated by the addition of either yeast extract, rice polishings, or thiamine. A marked variation in the requirements of nonpathogenic torulae for thiamine or its intermediates, thiazole and pyrimidine, were noted by Robbins and Kavanagh (1938). The ability of the organisms to utilize these substances for growth appeared to vary with the species. The pathogenic species, *Torulopsis (Cryptococcus) neoformans*, was not studied.

This organism is the occasional cause of a highly fatal form of meningitis, insusceptible, at present, to any form of therapy. Because of its importance as a human pathogen and the lack of information concerning its metabolism, this study was undertaken.

METHODS

Cultures. Six strains of *Torulopsis (Cryptococcus) neoformans*,[1] which had been isolated from fatal cases of *Cryptococcus* meningitis, were the microorganisms used in this study.

Media and methods. The basal synthetic medium, modified by the addition of various vitamins, was similar to that suggested by Georg (1948) and was as follows: asparagine, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.01 g; glucose, 5.0 g; Sørenson's

[1] Five of the cultures used in this study, torulae B, K, S, 968, and 1053, were kindly supplied by Miss R. A. Holt, Turo Hospital, New Orleans, Louisiana.

phosphate buffer mixture pH 6.8, 10 ml; purified agar, 1.5 g; and triple-distilled water, 90 ml. The final hydrogen ion concentration of the medium was pH 6.8. The agar was purified according to the method described by Robbins (1939). Although preliminary studies indicated that casein hydrolysate could replace asparagine in this medium, we found no advantage in making this substitution.

The various members of the vitamin B group tested were biotin, choline chloride, calcium pantothenate, inositol, nicotinic acid, riboflavin, and thiamine. Each member of this group was prepared separately in triple-distilled water, sterilized by Seitz filtration, and added aseptically to the medium after sterilization as required.

All chemicals were of cp grade or equivalent. All glassware and other materials used in the studies with the basal medium were immersed overnight in dichromate cleaning solution or in a suitable detergent. They were then washed free of the chemical with tap water and rinsed four times with triple-distilled water.

The Sabouraud medium used for comparative purposes had the following formula: Difco peptone, 1.0 g; glucose, 5.0 g; Difco agar, 1.5 g; distilled water, 100 ml; adjusted to a final hydrogen ion concentration of pH 6.8 with sodium hydroxide.

Both the synthetic and nonsynthetic media were added to test tubes in 4-ml amounts and so slanted as to provide an equal surface for growth in each tube. The standard inoculum per tube was 0.05 ml of a 4-day-old Sabouraud agar culture washed four times with triple-distilled water and standardized to a density of 50 on the Klett-Summerson photoelectric colorimeter using a no. 42 filter. Inoculated slants were incubated for 7 days at 30 C. This temperature was selected since it falls close to the optimum for the cultivation of the majority of pathogenic fungi.

At the end of the 7-day incubation period, each tube was observed for consistency, color, and density of growth. Density determinations were made with the Klett-Summerson photoelectric colorimeter after the growth was washed from each slant with 5 ml of distilled water and this suspension was diluted 1:20. All tests were run in triplicate so that the final reading in each case was an average of three single determinations.

EXPERIMENTAL RESULTS

Table 1 shows the growth obtained with six strains of *Torulopsis neoformans* on the basal synthetic medium with and without the addition of various members of the vitamin B complex. As mentioned previously, the density of growth was determined by the degree of transmission of light on the photoelectric colorimeter. The relationship of the density readings in the tables to the actual number of organisms, as determined by hemocytometer count, can be determined by reference to figure 1. Whereas the basal medium alone allowed a bare minimal growth with all strains, the addition of all of the vitamin B group, in 100 μg per 100-ml amounts each, gave a luxuriant mucoid growth. A similarly heavy growth was obtained when 100 μg per 100 ml of thiamine only was added. However, when all members of this complex except thiamine were incorporated

in the basal medium, growth fell to a level comparable to that on basal medium alone. That thiamine is the only member of the vitamin B complex stimulatory to the growth of T. neoformans is indicated by these results. It is also noteworthy that this synthetic medium suitably fortified with thiamine gave not only a heavier growth than that obtained on Sabouraud agar, but also a more uniformly characteristic growth of all strains. The moderate growth of the six strains on Sabouraud medium, with variation from a butyrous to mucoid con-

TABLE 1

Vitamin B requirements of T. neoformans on a basal synthetic medium as determined by density of growth

MEDIUM	T. NEOFORMANS STRAINS					
	A	B	K	S	968	1053
Basal	1	1	8	2	6	10
Basal plus 100 μg/100 ml B group*	138	139	103	104	142	134
Basal plus 100 μg/100 ml B group minus thiamine . .	1	3	1	8	1	2
Basal plus 100 μg/100 ml thiamine only	132	135	125	110	159	135
Sabouraud	50	58	39	55	40	42

* The term "B group" refers to the addition of choline chloride, calcium pantothenate, inositol, nicotinic acid, riboflavin, and thiamine in 100-μg amounts per 100 ml medium.
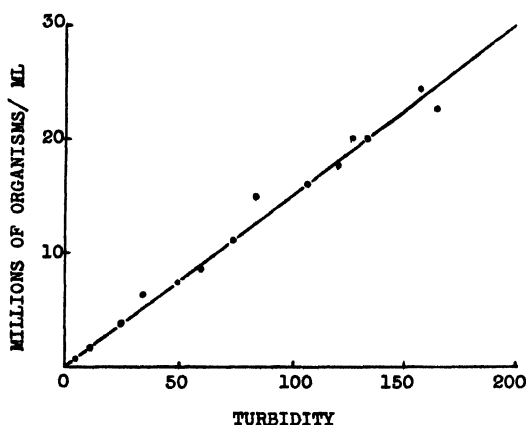
Figure 1. The relationship of turbidity readings on the Klett-Summerson colorimeter to the actual number of T. neoformans cells as determined by hemocytometer counts

sistency and cream to deep tan color, was in contrast to the uniformly heavy, mucoid, and cream-colored growth of these same strains on thiamine basal medium.

Certain of the variations in growth characteristics of a single strain of T. neoformans cultured on the media discussed above are shown in figure 2. Tube no. 9 shows the moderate, butyrous growth on Sabouraud agar. The scant growth in tubes no. 10 and no. 12 is characteristic for this organism in the basal medium

without thiamine. Tubes no. 11 and no. 13 show the heavy growth, so mucoid that it tends to run to the base of the slant, obtained when adequate concentrations of thiamine are added to the basal medium.
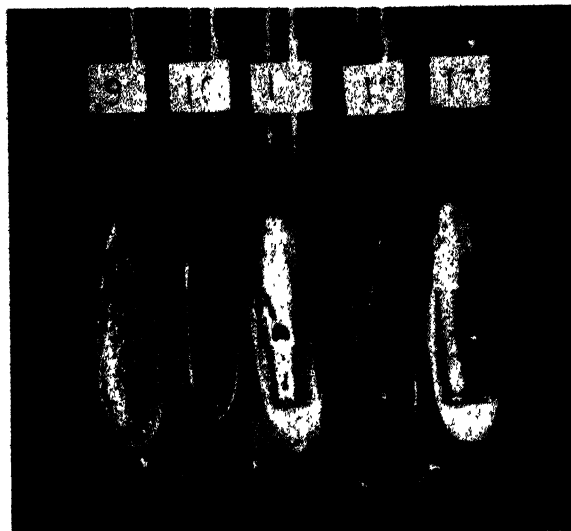
Figure 2. The variation in growth appearance of *T. neoformans* when cultivated in media differing in composition. No. 9: Sabouraud's medium. No. 10: Basal medium without vitamin B complex. No. 11: Basal medium plus 100 μg per 100 ml vitamin B complex. No. 12: Basal medium plus 100 μg per 100 ml vitamin B complex except thiamine. No. 13: Basal medium plus 100 μg per 100 ml thiamine only.

TABLE 2

Density of growth of T. neoformans on a basal medium as affected by concentration of thiamine

T. NEOFORMANS STRAINS	THIAMINE IN μG/100 ML				
	0.1	1.0	10	100	300
A	29	84	119	122	131
B	8	87	147	141	143
K	7	70	127	127	124
S	10	85	101	99	100
968	13	73	144	148	152
1053	16	90	120	117	127

Variation in the amount of thiamine in the medium markedly influenced the amount of growth of *T. neoformans* on this basal medium, as indicated in table 2. Concentrations of thiamine up to and including 1.0 μg per 100 ml were inadequate for producing maximal growth of *T. neoformans*. When concentrations of 10 μg per 100 ml were added, a maximal growth was obtained which was not increased by further additions up to 300 μg per 100 ml, the highest concentra-

tion tested. The effect of the addition of various concentrations of the other members of the vitamin B complex was not tested, since preliminary studies not included here, and the results given in table 1, had indicated that they were non-essential for the growth of this organism.

The comparatively poor growth of *T. neoformans* on Sabouraud medium, shown by the results given in table 1, indicated certain deficiencies in this medium for maximal and characteristic growth of this organism. In an attempt to determine what these essential nutritional components might be, the various ingredients of the basal synthetic medium were added separately to the Sabouraud formula. The results of this study are presented in table 3. Growth was not improved by the addition of magnesium sulfate or asparagine. The addition of phosphate buffer improved growth, as did also the addition of thiamine. In neither case, however, did the density equal that of the control basal medium plus thiamine. However, the addition of phosphate buffer and thiamine together to the Sabouraud medium resulted in a growth with all six strains equal to or exceeding

TABLE 3

Effect of a modified Sabouraud medium on the growth of T. neoformans

MEDIUM	T. NEOFORMANS STRAINS					
	A	B	K	S	968	1053
Sabouraud	42	39	43	47	28	57
Sabouraud plus 0.01% MgSO₄	47	44	42	51	33	53
Sabouraud plus 0.2% asparagine	43	49	39	55	28	51
Sabouraud plus PO₄ buffer	72	70	70	79	56	99
Sabouraud plus 100 μg/100 ml thiamine	—	79	—	78	75	71
Sabouraud plus PO₄ buffer and 100 μg/100 ml thiamine	120	160	154	138	176	165
Basal plus 100 μg/100 ml thiamine	98	135	140	130	147	159

that of the control, thiamine basal medium. This growth also became more mucoid in character. This supplemented Sabouraud medium thus became suitable for the production of maximal and characteristic growth of *T. neoformans*.

SUMMARY

The essential nutrient requirements for the growth of *Torulopsis neoformans* with particular reference to the vitamin B complex have been determined. Thiamine was found to be the only member of the vitamin B group tested that was stimulatory to the growth of this microorganism.

A synthetic medium suitable for the cultivation of *T. neoformans* has been suggested that has the advantage of reproducibility and also gives much heavier and more characteristic growth than that obtained on the Sabouraud medium.

The addition of phosphates and thiamine to the Sabouraud formula made this deficient medium suitable for the maximal growth of *T. neoformans*. The stimulation to the growth of *T. neoformans* brought about by the addition of these two components either to a synthetic medium or to Sabouraud medium suggests

the possibility that the function of the vitamin here is that of cocarboxylase. However, this problem has still to be investigated.

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STIMULATION OF THE GROWTH OF A STRAIN OF CORYNEBACTERIUM DIPHTHERIAE BY POLYVINYL ALCOHOL[1]

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Certain strains of *Corynebacterium diphtheriae* require, for optimal growth from small inocula, protein containing material such as animal serum, milk, or crude casein in addition to the customary amino acids, salts, vitamins, and carbohydrates (Cohen, Snyder, and Mueller, 1941). Other organisms have analogous requirements, either for proteins or other naturally occurring compounds of high molecular weight, such as starch (Dubos, 1947; Ley and Mueller, 1946; Sloane and McKee, 1949). The possibility exists, however, that the apparent activity of natural products such as protein may be due to some unrecognized contaminant. Efforts, therefore, have been made to replace the crude proteins of our earlier experiments by purer materials such as crystalline bovine serum albumin and by synthetic, water-soluble polymers of high molecular weight. It is the purpose of this paper to present data on one such polymer, polyvinyl alcohol, which show it to be an effective substitute for protein in the case of one strain of *C. diphtheriae*.

MATERIALS, METHODS, AND PROCEDURE

Basal medium. This was prepared daily from the following stock solutions:

A. 20 g casein hydrolyzate[3] 3 ml ethyl alcohol (95%)
   50 ml NaCl (20%) 11 ml DL-lactic acid[4] (85%)
   50 ml Na_2HPO_4 (8%) 0.35 ml nicotinic acid (1%)
   50 ml KH_2PO_4 (1.2%) 0.7 ml pimelic acid (0.1%)
   50 ml $MgCl_2 \cdot 6H_2O$ (1.2%) 7.0 ml calcium pantothenate (0.1%)
   5 ml glycerol 0.5 ml phenol red solution (0.6%)
   Final volume—258 ml

B. Cystine—1 per cent solution in 97 ml H_2O plus 3 ml concentrated HCl.

C. Sodium oleate—prepared weekly by dissolving 0.1 ml oleic acid in 9.9 ml of H_2O plus 1 drop of 40 per cent NaOH. A portion was diluted daily in water so that the required amount, 5.0×10^{-5} ml, was present in about 0.5 ml of solution.

D. Tryptophan—0.1 per cent solution, prepared weekly in 10-ml amounts with the aid of 1 drop of 40 per cent NaOH.

The basal medium for each plate consisted of (A) 1.8 ml, (B) 0.56 ml, (C) 0.5 ml, (D) 0.1 ml, 5 per cent agar 7 ml, HCl or NaOH to the neutral point of phenol

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[1] Aided by a grant from the Patrons of Research, Beth Israel Hospital.
[2] With the technical assistance of Bernice Shafran.
[3] "Casamino acids" (Difco), total nitrogen 10 per cent, sodium chloride 14 per cent.
[4] Neutralized with NaOH.

red, the material under test, and water to 20 ml. Liquid medium consisted of one-half the foregoing quantities plus water and material under test to 10 ml.

Polyvinyl alcohol. Polyvinyl acohols[5] of different viscosities were tested. All had similar activity, but for convenience relatively low viscosity products — "elvanol" type A, grades 51-05 and 70-05—were used for most of the work. They were added to the basal medium either as a 10 per cent solution in water or as the dry powder. Sterilization was by autoclaving as usual, since such material showed no less activity than media sterilized by Seitz filtration.

Inoculum. The test organism was a *gravis* strain of *C. diphtheriae*. It was subcultured approximately every 2 weeks in defibrinated human blood and stored in the refrigerator between subcultures. For each day's inoculum the tip of a straight wire was dipped in the blood culture and then transferred to 10 ml of sterile water. One drop of this dilution was used immediately for inoculation of each plate. The number of organisms in each inoculum was 10 to 200, as judged by colony counts.

TABLE 1

Effect of protein supplements

	SOLID MEDIUM DIAMETER OF COLONY IN MM		LIQUID MEDIUM MG BACTERIAL N AT 64 HOURS
	16 hours	40 hours	
Basal medium..................................	Trace	1.3	0
Basal + horse serum, 0.5 ml*.................	0.6	3.0	2.35
Basal + crystalline bovine albumin, 50 mg....	0.3	4.0	+++†
Basal + casein, 50 mg	0.5	4.2	+++

* The quantities cited in all tables are those for 10 ml of final medium.

† A large amount of growth, comparable to that obtained with horse serum.

Performance of test. Tubes were autoclaved at 10 pounds for 10 minutes, and plates were poured from the hot tubes. Protein solutions, other than casein, were sterilized separately by Seitz filtration and added to the tubes after the latter had cooled to 45 C. One drop of the dilute inoculum was spread over the surface of each plate. The temperature of incubation was 37 C. Growth was recorded on solid media by measuring the colonial diameter at 16 and 40 hours, on liquid media by estimating the size of the pellicle at 64 hours. Significant differences in growth in liquid media were checked by determination of the total nitrogen of the bacteria collected and washed by centrifugation.

RESULTS

Growth of the test strain of *C. diphtheriae* in the fluid basal medium was negligible, and in the agar basal medium was poor and delayed. Supplements of horse serum, casein, crystalline bovine albumin, or polyvinyl, alcohol caused distinct stimulation of growth (tables 1 and 2). Earliest growth regularly oc-

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[5] We are indebted to Mr. L. S. Litchfield of E. I. du Pont de Nemours Company for supplies of polyvinyl alcohol, trade name "elvanol."

curred in media containing serum, although other supplements yielded about the same amount of growth by the time of the conclusion of the experiment. Growth on the solid polyvinyl alcohol media was slightly delayed in comparison with horse serum media, but, between 16 and 40 hours, it was rapid and regularly yielded flat, very large colonies (figure 1). Liquid media containing polyvinyl alcohol usually yielded results like those tabulated; very rarely they failed to support any growth. The combination of serum or bovine albumin and polyvinyl alcohol yielded no increase in growth over that produced by the individual supplement alone. The effects, therefore, are not additive and may well be of a similar nature.

The casein hydrolyzate represented the only chemically undefined material in the basal medium. The possibility was considered that protein and polyvinyl

TABLE 2

Effect of polyvinyl alcohol

	SOLID MEDIUM DIAMETER OF COLONY IN MM		LIQUID MEDIUM MG BACTERIAL N AT 64 HOURS
	16 hours	40 hours	
Basal medium	Trace	1.0	0
Basal + horse serum, 0.5 ml	0 75	2 6	2.35
Basal + polyvinyl alcohol, 12 5 mg	Trace	2.0	—
Basal + polyvinyl alcohol, 25 mg	Trace	2.2	0
Basal + polyvinyl alcohol, 50 mg	0 25	3.0	Trace
Basal + polyvinyl alcohol, 100 mg	- -	--	1.14
Basal + polyvinyl alcohol, 125 mg	0 3	4.2	---
Basal + polyvinyl alcohol, 250 mg	0 3	3.2	--
Basal + polyvinyl alcohol, 300 mg		--	1 59
Basal + polyvinyl alcohol, 350 mg	0 3	3 0	—
Basal + polyvinyl alcohol, 425 mg	0 5	3.5	---
Basal + polyvinyl alcohol, 500 mg	0.3	3 5	1 64

alcohol acted by neutralizing some hypothetical toxic material in the casein hydrolyzate. Therefore, the minimal number of pure amino acids, in concentrations found to be optimal for the test organism, was substituted for casein hydrolyzate. The stimulating effect of serum and of polyvinyl alcohol was still observed, although the absolute amount of growth was less (table 3).

Efforts were made to fractionate the commercial polyvinyl alcohol in the hope of obtaining more potent preparations. These attempts were unsuccessful and need not be cited in detail; they were, however, of some interest since they demonstrated that the growth-promoting capacity of polyvinyl alcohol was readily reduced or destroyed. In general, extraction of polyvinyl alcohol itself with organic solvents (ether, ethyl alcohol) yielded no appreciable activity in the solvent phase and unchanged activity in the polymer. Similarly, extraction of aqueous solutions of polyvinyl alcohol with an immiscible solvent (ethyl ether) yielded no change in activity. Ten per cent aqueous solutions of polyvinyl alcohol retained undiminished activity after 2 weeks at room temperature. How-

Figure 1 Number 1.
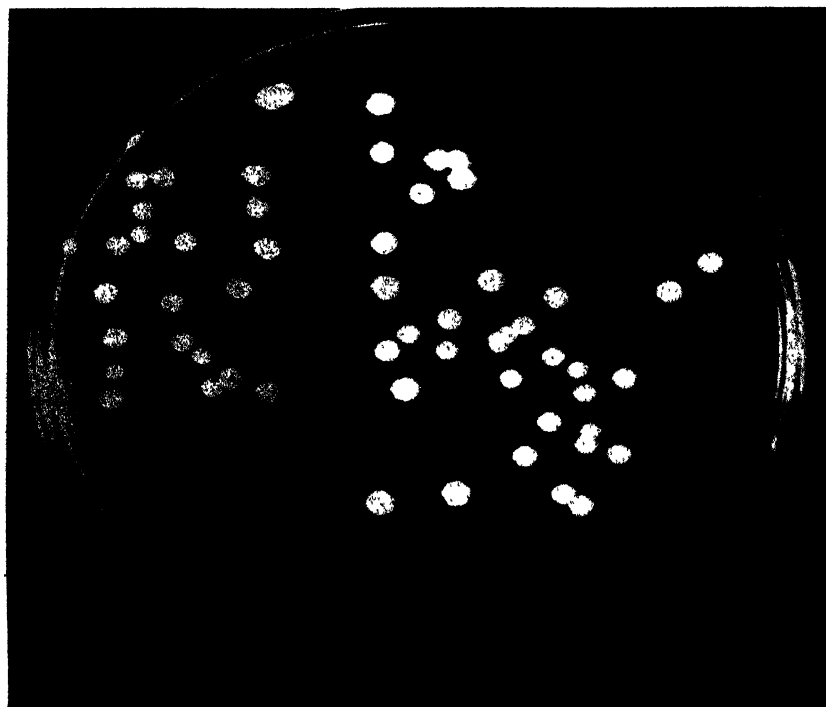
Figure 1 Number 2.
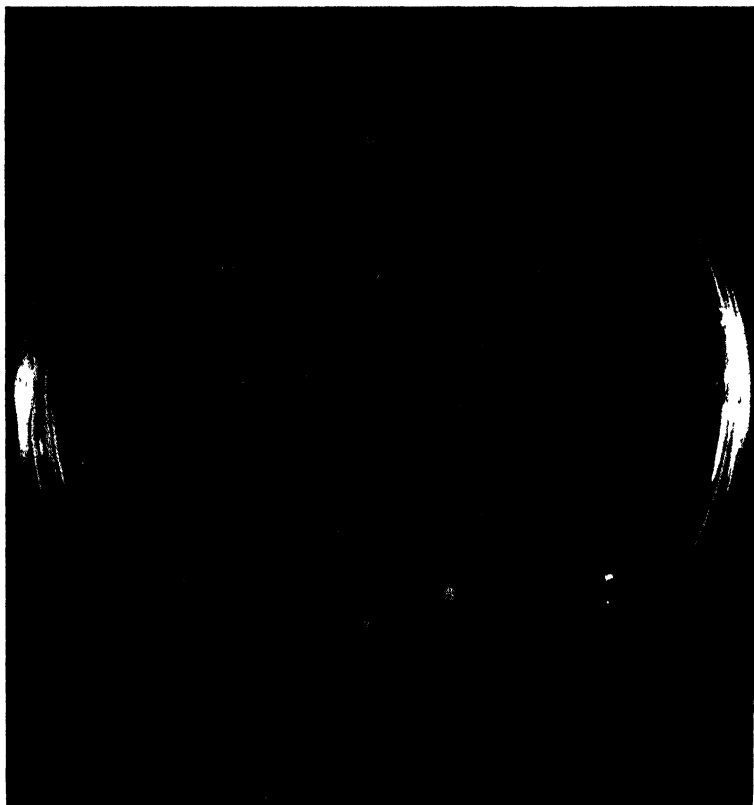
Figure 1 Number 3.

Figure 1 The stimulating effect of protein or polyvinyl alcohol on the growth of a *gravis* strain of *C diphtheriae*, 40 hours' incubation No 1 Basal medium Colonies average 1 mm in diameter No 2 Basal medium plus 1 ml horse serum Colonies average 3 2 mm in diameter No 3 Basal medium plus 1 g polyvinyl alcohol Colonies average 5 mm in diameter

TABLE 3

Effect of substitution of a few amino acids for casein hydroyzate

	DIAMETER OF COLONIES IN MM	
	16 hours	40 hours
Basal medium*	0	0 6
Basal + polyvinyl alcohol, 125 mg	0	3 8
Basal + polyvinyl alcohol, 250 mg	0	3.8
Basal + polyvinyl alcohol, 350 mg	0	3 5
Basal + polyvinyl alcohol, 500 mg	0	3.8
Basal + horse serum 0.5 ml	0.5	2 5
Basal + casein 50 mg	Trace	3.5

* Without casein hydrolyzate but with the addition of DL-valine, 7.5 mg; DL-isoleucine 5 mg; DL-methionine, 2 5 mg; L-glutamic acid, 100 mg.

ever, precipitation of polyvinyl alcohol from aqueous solutions with miscible solvents such as acetone, dioxane, or ethyl alcohol yielded fractions partially or completely inactivated. Indeed, even concentration of a dilute aqueous solution by distillation *in vacuo* at the water pump caused loss of activity. The inactivation is not referable to heating since polyvinyl alcohol is not inactivated by autoclave temperature. Partial inactivation is also induced by dialysis of polyvinyl alcohol solutions against tap or distilled water (table 4). The possibility of recovering activity by combining the dialyzate and nondialyzable material could not be tested in view of the inactivation caused by the concentration of a large volume of polyvinyl alcohol solution.

TABLE 4

Effect of dialyzed polyvinyl alcohol

	MG BACTERIAL N AT 64 HOURS	
	Undialyzed	Dialyzed[†]
Basal medium*	0	0
Basal medium + polyvinyl alcohol, 25 mg	0	0
Basal medium + polyvinyl alcohol, 100 mg	1.14	0.16
Basal medium + polyvinyl alcohol, 300 mg	1.59	1.60
Basal medium + polyvinyl alcohol, 500 mg	1.64	1.97

* This sample of basal medium contained pyruvic acid, 0.1 ml, and lactate, glycerol, and alcohol were omitted.

† Dialyzed for 3 days in cellophane against running tap water.

The chemistry of polyvinyl alcohol indicates a linear structure with terminal aldehyde groups (Marvel and Denoon, 1938; Marvel and Inskeep, 1943):

$$-CH_2-CH-CH_2-CH^6-$$
$$OH \qquad OH$$
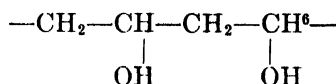
Substances of somewhat similar or related chemistry were tested with negative findings. Included were these compounds: starch, "carbowax,"[7] sodium carboxy methyl cellulose,[8] vegetable gums (arabic, matti, tragacanth, acacia), citrus pectin, acetate, acetaldehyde, and glucose.

DISCUSSION

These data indicate that commercial preparations of polyvinyl alcohol stimulate the growth of small inocula of a *gravis* strain of *C. diphtheriae* in a synthetic medium. The rapidity and quantity of growth were slightly inferior to that produced by horse serum, casein, or crystalline bovine albumin. The explanation for this phenomenon is obscure. However, a similarity is suggested to the effects

[6] Analytical data supplied by the manufacturers indicate the presence of traces of sodium acetate, methanol, methyl acetate, methyl benzoate, and sodium benzoate or benzoic acid.

[7] Polyethyleneglycol, supplied by Carbide and Carbon Chemicals Corporation.

[8] Supplied by E. I. du Pont de Nemours Company.

of starch in the case of *Neisseria* (Ley and Mueller, 1946) and albumin in the case of *Mycobacterium tuberculosis* (Dubos, 1947). These materials have been shown to function through the adsorption of toxic fatty acids, particularly oleic acid. Accordingly, solutions of polyvinyl alcohol were tested for their ability to adsorb oleate, using oleate-induced hemolysis of red blood cells as an indicator of free oleate. No evidence of adsorption was found. The possibility is open that polyvinyl alcohol may adsorb other substances from the medium, which may be present in concentrations toxic for initial growth. The adsorptive ability of polyvinyl alcohol has been found, for example, to be high for inorganic ions (Herrman and Haehnel, 1927).

The frequency with which polyvinyl alcohol was inactivated by relatively mild physical and chemical treatment was unexpected. Many of these procedures, however, produce changes in the physical properties of the polyvinyl alcohol. Marvel has shown the ready occurrence of interreactions (acetal formation, aldol condensation) among the polymeric chains. It is possible that the procedures employed produced enough such reactions in the starting material to render it less active biologically.

The question arises whether polyvinyl alcohol functions as a nutrient and is metabolized by *C. diphtheriae*. That some of the carbon atoms of polyvinyl alcohol are susceptible of enzymatic oxidation has been demonstrated in the case of preparations from *Fusarium lini* (Nord, Damman, and Hofstetter, 1936; Damman, Lange, Bredig, and Nord, 1936). However, it does not seem reasonable that *C. diphtheriae* should prefer such an unnatural compound as a carbon source to the exclusion of many other naturally occurring compounds.

Additional growth factors for *C. diphtheriae* have been described (Chattaway, Dolby, Hall, and Happold, 1948). Those appear to be mixtures of amino acids and peptides and present no obvious connection with the material of this paper.

SUMMARY

Polyvinyl alcohol, a synthetic, water-soluble polymer, was found to stimulate the growth, in a synthetic medium, of small inocula of a *gravis* strain of *Corynebacterium diphtheriae*.

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CHARACTERIZATION OF THE AGGLUTINATING PRINCIPLE IN CHORIOALLANTOIC FLUID RESPONSIBLE FOR THE CLUMPING OF CERTAIN STRAINS OF STAPHYLOCOCCUS AUREUS[1]

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Chorioallantoic fluid from fertile hens' eggs possesses the ability to agglutinate certain strains of *Staphylococcus aureus* when these organisms are grown in the presence of this material (Shrigley, 1945). It has also been found that fluids from eggs infected with the influenza virus (PR8) possess this agglutinating property in a significantly higher degree than do normal fertile egg fluids. It was felt, therefore, that the identification of this principle was of importance, as its nature might shed some light on the effect of the infectious agent on the metabolic economy of the cells involved.

The present article is a more detailed consideration and an extension of observations presented in a preliminary report concerning this phenomenon (Shrigley and Maculla, 1947).

MATERIAL AND METHODS

Most of the observations recorded below have been made on normal chorioallantoic fluids. After comparison of these with the experiments conducted with influenza-virus-infected materials, however, it was concluded that the agglutinating principles in the two fluids differ quantitatively and not qualitatively.

Chorioallantoic fluids were obtained from eggs of hens of the Rhode Island Red breed, the eggs being available from a local hatchery. Following 11 to 12 days' incubation at 103 F in a "humidaire" incubator, the eggs were placed for 2 to 3 hours at 4 C and then harvested in the manner already described (Shrigley, 1945). The material was pooled and stored in 20-ml lots at 4 C until a volume of 500 or 1,000 ml had accumulated. Under these conditions of storage the principle remained active for several months.

The strain of influenza virus (PR8) used was the same as that mentioned in the 1945 report, and in addition an egg-adapted strain of PR8 was employed.[3] The methods of inoculating the eggs and the harvesting of the virus fluids were those commonly used in the cultivation of this agent, and have already been

described in a previous paper. In all cases the presence and potency of the virus were determined by the chicken red blood cell agglutination technique as described by Hirst (1942).

Tests for the bacteria-agglutinating properties of the materials studied were conducted as previously described. Alkaline extract broth (pH 8) was used as a medium and diluent for the various fluids and their fractions under observation. To 1 ml of each of the fluid dilutions, 0.1 ml of an 8- to 10-hour culture of an agglutinable strain of *Staphylococcus aureus* was added and the mixture incubated overnight at 37.5 C. All of the tests for agglutination were made with the Craig strain (Shrigley, 1945). At times the Wood 46 strain was used as a negative control, since these organisms are not agglutinated by the principle in the chorioallantoic fluid.

The studies of the various properties of the agglutinating principle to be described below were made on crude chorioallantoic fluid as well as on fluids that had undergone concentration and partial purification.

TABLE 1

The increase in bacterial agglutination titer of chorioallantoic fluid as a result of concentration by filtration under pressure

| | DILUTION OF EGG FLUIDS IN NEGATIVE POWERS OF 2 | | | | | | | | | | |
	2	3	4	5	6	7	11	12	13	14	Control
Fluid before concentration											
Normal..........	++++	++++	+++	++	+	—					—
Virus-inf........	++++	++++	++	+	—	—					
After concentration											
Normal..........	++++	++++	..	..	..	..	++++	++++	++	—	—
Virus-inf........	++++	++++	..	..	..	..	++	+	—	—	—

RESULTS

Concentration of the agglutinating principle. Of the several different methods tried in an effort to concentrate the staphylococcus-agglutinating principle in the chorioallantoic fluid, two were found to be of practical value. Lyophilization of the crude fluid followed by resuspension of the residue in one-tenth the original volume of sterile distilled water was the technique used for studies on the solubility of the principle in various fat solvents.

The second method of concentration has proved more useful. This consists of filtration of the chorioallantoic fluid under pressure through collodion membranes. Since the agglutinating principle is not dialyzable, it does not pass through this material. Fluids from several harvests were pooled to make 500- to 1,000-ml volumes, and filtration was done in the cold (4 C) under 500-mm pressure of mercury. Following this procedure, which usually took from 24 to 48 hours, the residue was dialyzed overnight in cold running tap water and then dialyzed for another 24 hours in phosphate buffer pH 7.7 at 4 C. Table 1 gives

examples of the degree of concentration of bacterial agglutinating principle that may be achieved by this method. Of the many samples tested, not one showed the presence of the bacterial-agglutinating property in the fluid that passed through the membranes.

At the lower concentrations there was a closer correlation between the degree of concentration of the fluid and the increase in bacterial agglutinin titer. On the other hand, for the more highly concentrated material it appeared that some of the activity was lost during the concentration process. This is illustrated below:

Number of times concentrated	Number of times titer increased
5	4
9	8
10	4.5
17	8
50	32
60	16
100	35
150	64

When attempts were made to concentrate larger volumes of fluid, a longer period of time was required for the concentration and therefore it is possible that more loss of activity was incurred because of the instability of the principle under these conditions.

The amount of protein nitrogen in the dialyzed concentrate varied with the degree of concentration achieved and with the amount of protein present in the original fluid. Of this original protein it was found that only a small fraction possessed the agglutinating activity. The nitrogen values in the concentrated material varied from 0.12 ± 0.04 mg per ml after a concentration of 10 times, to 0.97 ± 0.13 mg per ml after a concentration of 100 times.

In spite of the increase in the agglutination titer of the chorioallantoic fluid as measured by the Craig strain, at no time did the concentrated material agglutinate the nonagglutinable Wood 46 organisms. On the other hand, it was of interest to note that occasionally fluids that would not agglutinate Craig at a dilution of 1:4 would do so after being concentrated 10 times.

Defining the Properties of the Agglutinating Principle

Solubility in various solvents. The methods used in this study involved several procedures. Attempts were first made to extract the agglutinating principle by treating crude lyophilized material with acetone and alcohol. On the other hand, ether, being immiscible with chorioallantoic fluid, was added directly to the crude liquid. Another method consisted of applying the various cooled fat solvents to the chilled residues obtained from crude fluids that had been allowed to dry by evaporation at 37 C. Solvents were allowed to stand in contact with their respective samples for 10 minutes before separation. Following separation, all fractions were evaporated at 4 C before resuspension of the residues. The residues, both soluble and insoluble, were resuspended in sterile distilled water

if the fluids had originally been lyophilized, and in alkaline nutrient broth if they had been dried by evaporation. All extractions were made in the cold.

Observations made on highly concentrated and purified preparations of the agglutinating principle confirm in every detail those made with the crude material. The findings are consistent with the fact that the agglutinating principle is not soluble in chloroform, alcohol, ether, or acetone under the conditions of these studies.

The effect of heat. Although it has been reported that the agglutinating principle is thermolabile (Shrigley, 1945), it was considered of interest to examine more fully the rates of inactivation of this material at various temperatures. For this study 2-ml samples of crude fluid were placed in each of three tubes and all placed in a thermostatically controlled water bath. At various time intervals, depending on the temperature being studied, the tubes were removed and the contents titrated for agglutinating activity. The time periods between sampling

TABLE 2

The mean velocity constants of heat of inactivation of the agglutinating principle at various temperatures

	TEMPERATURES, C						
	46	45	44	43	42	41	40
Mean velocity constants = k......	0.41	0.11	0.048	0.030	0.020	0.010	0.010
Number of observations used......	2	5	5	5	9	4	6
k_I/k_{40}*	41.0	11.0	4.8	3.0	2.0	1.0	1.0
log k_I/k_{40}	1.61	1.04	0.68	0.48	0.30	0.00	0.00

*Ratio of velocity constant for a particular temperature to the velocity constant at 40 C.

were shorter, from necessity, for the higher than for the lower temperatures. No attempt was made to keep the pH constant during these studies. It was felt that the observations on inactivation at higher temperatures possessed a larger intrinsic error than those involving less heat, since inactivation took place so promptly that it was possible the temperature of the fluid in the test tube did not reflect the true temperature of the water bath. For lower temperatures this was not a factor since at 45 C and less no samples were taken before 5 minutes after contact of the fluid with bath temperature. The temperatures studied ranged from 40 C to 46 C.

On the whole, the results from different samples of egg fluid varied considerably as to their rate of inactivation. However, it was found consistently that the agglutinating principle was quickly destroyed at 46 C and slowly inactivated at 40 C. On the other hand, the percentage of activity at various times during exposure to these temperatures was not always uniform. For example, some fluids were reduced in strength at the particular temperature in question but never became completely inactivated during the time studied. The causes of this variation may have been several. Possibly, as stated in earlier studies, the original quantity of agglutinating principle present in the crude material was a

factor in its destruction. Support for this suggestion was seen in that, in general, the concentrated, purified material seemed more resistant to heat than the crude fluid. Further, the experimental error in determining the quantity of agglutinating principle remaining after heating is an exceedingly important variable. By the method of titration used here it was arbitrarily considered that this error was as much as plus or minus one power of 2, the serial dilutions being by halves. Attempts were made to control these variables and data were obtained which, though not large in number, suggested a definite trend.

Table 2 shows the mean velocity constants for fluids heated at various temperatures. These constants were obtained by substituting in the equation[4] describing the rate of a first-order reaction the value of the highest dilution of the heated fluid which gave agglutination of staphylococci and by contrasting it with the highest dilution of unheated fluid which caused clumping. All observations presented are on crude chorioallantoic fluid.

Figure 1 represents the relation between the logarithm of k_t/k_{40} for the various temperatures studied: k_t is the velocity constant for the specific temperature under consideration, and k_{40} represents a similar expression for 40 C. From these determinations it is possible to obtain an approximation of the amount of heat energy necessary to inactivate the agglutinating principle. This figure was obtained from the following relationship:

$$E_A = \frac{4.58 \, T_1 \, T_2}{T_2 - T_1} \, \text{Log} \, k_2/k_1$$

where E_A represents the activation energy of the inactivation process, T_1 and T_2 the absolute temperatures studied, and k_1 and k_2 the respective velocity constants. The 4.58 is the product of the gas constant, 1.987, and the conversion factor, 2.303 (Höber, 1945). E_A was found in these experiments to be of the order of 120 kilocalories per mole.[5]

The effect of ultraviolet light. Freshly harvested, crude chorioallantoic fluid was exposed to the ultraviolet light from a mercury-arc tube at a distance of 14 cm. Three samples from the treated fluid were removed and tested, one at 30, one at 45, and one at 60 minutes. There was no evidence of any destruction of the agglutinating principle even after the 60-minute exposure.

Attempts at purification of the agglutinating principle. Pooled normal chorioallantoic fluids as well as those that were influenza-virus-infected were concentrated by ultrafiltration as described above and then subjected in the cold to fractional precipitation with ammonium sulfate. During this fractionation the pH

[4] The authors acknowledge with thanks the help of Dr. Ernest C. Pollard in pointing out this approach to the problem of heat inactivation and in giving aid with the calculations. The velocity constants were obtained by solving for k in the equation $I/I_0 = e^{-kt}$ in which I equals the titer of the fluid after heating for a time t at a specific temperature, and I_0 is the titer of the fluid before heating, and e is the base of the Napierian system of logarithms.

[5] For example, from the line in figure 1 we may read directly the values for Log k_t/k_{40} at 46 C, and at 41 C. Substituting in the equation above we get $E_A = \left(\dfrac{(4.58)(319)(314)}{5} \right)$ (1.37−0.07). Solving this one gets E_A to equal 120 kilocalories per mole.

was held at 7.7. The agglutinating activity appeared to be precipitated max-imally when the concentration of ammonium sulfate was at $\frac{1}{4}$ saturation. A minimal amount of the activity was occasionally present in the precipitate that was obtained when the ammonium sulfate concentration was brought to $\frac{1}{2}$ saturation. Precipitates that formed by the addition of higher concentrations of the salt did not yield any agglutinating activity. The properties of the original normal fluids and those of their various fractions may be seen in figure 2.
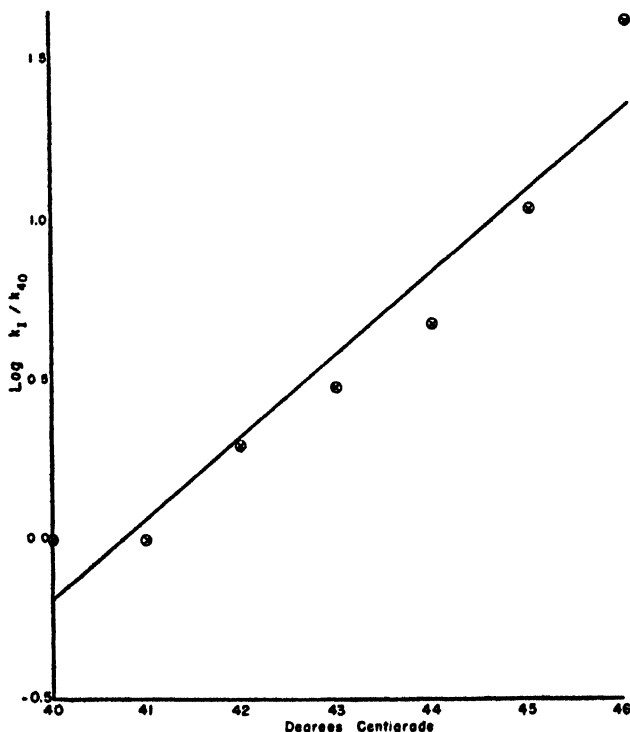
Figure 1. The ratio of the mean velocity constants of inactivation of the agglutinating principle at various temperatures with respect to k_{40}. The mean activation energy of inac-tivation equals 120 kilocalories per mole.

Although most of the observations on the chemical properties of the agglu-tinating substance were made on normal chorioallantoic fluid, there were also some data obtained from the study of influenza-virus-infected material. As indicated by the chicken red blood cell agglutination technique, the majority of the virus was precipitated from the concentrate by the conditions of $\frac{1}{4}$ and $\frac{1}{2}$ ammonium sulfate saturation. At $\frac{3}{4}$ ammonium sulfate saturation only a small amount of the agent was precipitated; none was recovered at complete satura-tion.

The effect of proteolytic enzymes on the agglutinating principle. In an effort to determine further the nature of the agglutinating principle, the effect of crystal-

line trypsin[6] on the potency of the material was studied. As a control, the activity of the enzyme was tested by measuring its ability to hydrolyze fibrinogen. The tests were conducted at pH 7.7 by adding an equal volume of trypsin, diluted 1:1,000 by weight in phosphate buffer, to 0.5 ml of a solution of the concentrated agglutinating principle and incubating the mixture at 37 C. Samples were taken and titrations made immediately after mixing enzyme and substrate, and further samples were removed for study at 5- and 10-minute intervals for 1 hour. The
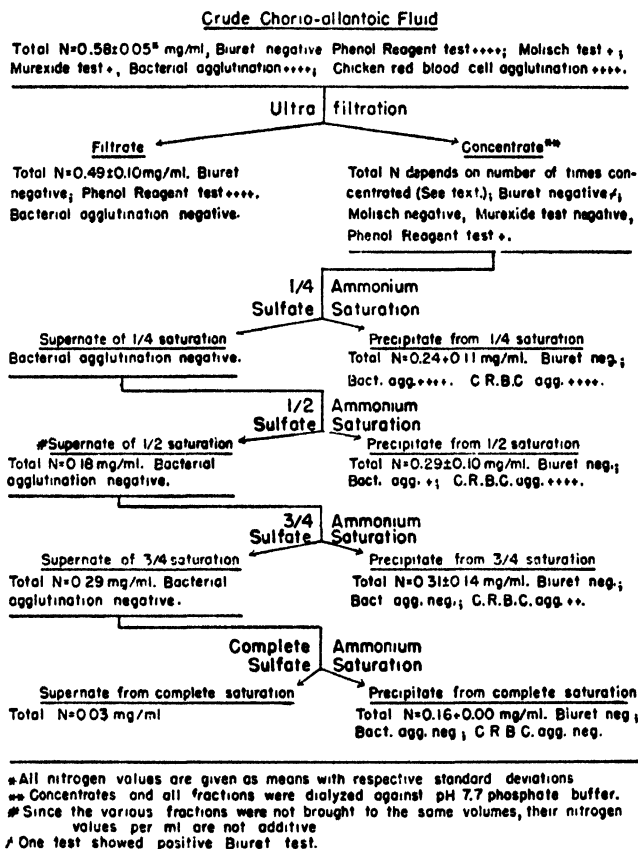
Figure 2. The summary of some properties of normal and influenza-virus-infected chorio-allantoic fluids and their fractions.

final dilution of the trypsin in these tests was 1:2,000. The enzyme was not inactivated in the various samples before they were removed for titration of the agglutinating principle, since it was feared that if this were done the agglutinating principle might also be damaged. Further, it was considered that since the dilution of the trypsin in the first tube of the titration was 1:8,000, it would be

[6] It is a pleasure to acknowledge the generosity of Doctor Downie, of the Department of Surgery, Yale University School of Medicine, in furnishing us with crystalline trypsin.

very unlikely that the inhibiting effect observed would be the result of the enzyme acting directly on the bacteria themselves rather than on the agglutinating principle. Studies were made on crude as well as purified agglutinating principle and, on the whole, the results were in agreement. The data, however, were conclusive only when the concentrated, relatively pure principle was used.

It was found that crystalline trypsin in a concentration of 1:2,000 destroyed the agglutinating principle almost immediately on mixing, and there was definitely no ability on the part of the fluid to clump staphylococci after 5 minutes of contact with the enzyme at 37 C. The controls, which consisted of heated enzyme plus concentrated principle, and of concentrated principle alone, showed no diminution of titer during the whole experiment. Further controls were also made using crystalline enzyme plus staphylococci, and no agglutination was observed here. The studies undertaken with pepsin did not yield clear-cut results. Since crude enzyme preparations only were available, the activity of this enzyme was not studied in detail.

Migration of principle in an electric field. For the past year attempts have been made to concentrate the agglutinating principle sufficiently so that it might be studied in the Tiselius apparatus. We have reported observations on crude chorioallantoic fluid which showed that the principle migrated to the positive electrode (Shrigley and Maculla, 1947). However, the boundaries indicated, as one might expect, that the material was not homogeneous. In our apparatus it is necessary to have at least 5 mg of total protein in a volume of 5 ml in order to get a boundary. We have never been able to meet these requirements by our methods of concentration and purification.

Spectrophotometric studies of the agglutinating principle. Figure 3 illustrates the ultraviolet absorption curves of concentrated influenza-virus-infected chorioallantoic fluid, as well as the absorption spectra of various fractions from concentrated normal fluids precipitated by different amounts of ammonium sulfate. All samples were dialyzed in pH 7.7 phosphate buffer and then diluted so that they contained 0.03 mg of nitrogen per ml before testing. It can be seen that in the 275- to 280-mμ region the absorption by the concentrated, unfractionated virus-infected material dropped from a plateau which extended from 255 mμ. The absorption of the respective resuspended precipitates obtained from the $\frac{1}{2}$ and $\frac{3}{4}$ ammonium sulfate saturation of concentrated normal chorioallantoic fluid possessed similar characteristics. Special attention should be directed to the absorption curve of the resuspended precipitate recovered by the $\frac{1}{4}$ saturation with ammonium sulfate of normal, concentrated fluid. This fraction possessed the greatest amount of bacteria-agglutinating activity. From figure 3 it may be seen that there is a definite absorption in the 260-mμ region and a minimum at about 245 to 250 mμ. Although not all resuspended precipitates from this fraction showed this pattern exactly (figure 4), the majority gave a comparable picture.

Fractions of the normal chorioallantoic fluids obtained from the $\frac{1}{4}$ saturated ammonium sulfate solutions that had for one reason or another lost their ability
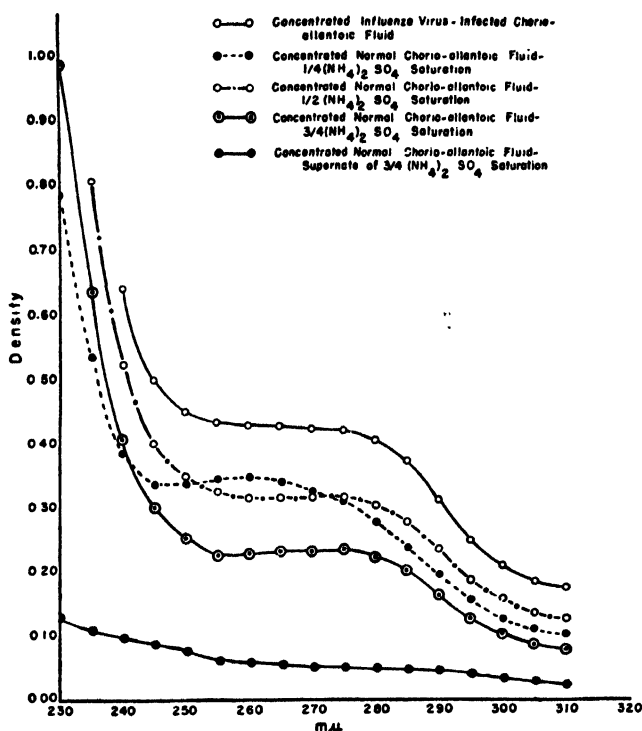
Figure 3. The ultraviolet absorption curves of concentrated virus-infected chorioallantoic fluid and various ammonium-sulfate-precipitated fractions of concentrated normal chorioallantoic fluid. All fluids were adjusted to 0.03 mg of N per ml. Dialysis was against pH 7.7 phosphate buffer.
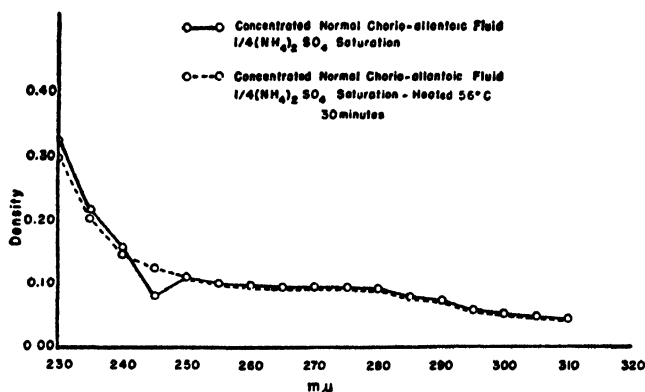
Figure 4. The ultraviolet absorption curves for the fraction of normal concentrated chorioallantoic fluid precipitated by the addition of ¼ saturation of ammonium sulfate, and the effect of heating this fraction at 56 C for 30 minutes. All fluids were adjusted to 0.03 mg of N per ml. Dialysis was against pH 7.7 phosphate buffer.

to agglutinate staphylococci, were also studied spectrophotometrically. The absorption curves in the ultraviolet of these inactive samples differed from the spectra of the active ones. The outstanding alterations seen were the increase in absorption in the 245- to 250-mμ region and the decrease in the 260-mμ area. The former change is most clearly demonstrated in the experiment, illustrated in figure 4, in which the active fraction had been inactivated by heating to 56 C for 30 minutes. Other samples that had been allowed to stand at 4 C for several days, if undiluted, retained their original absorptive properties. On the other hand, if diluted with phosphate buffer pH 7.7 so that they contained 0.03 mg of nitrogen per ml and then allowed to stand at 4 C, the agglutinating activity decreased and there was alteration in the absorption patterns. Figure 4 is also presented to show that the absorption spectra of all $\frac{1}{4}$-saturated fractions were not uniform. This is interpreted as an indication that the precipitates obtained by this procedure are not always composed of the same materials even though this fraction does always contain the agglutinating principle. Finally the supernatant of the $\frac{1}{4}$ fraction possessed almost no material that absorbed light in the ultraviolet region.

<div align="center">DISCUSSION</div>

Because it was observed that the staphylococcus-agglutinating principle in the chorioallantoic fluid of hens' eggs was of greater potency in influenza-virus-infected material than in normal fluid (Shrigley, 1945), studies have been made in an attempt to characterize more fully the properties of the principle. It was hoped that a better knowledge of these properties might contribute something to an understanding of the complex processes of virus infection. Although the chemical nature of this substance responsible for the clumping of some strains of staphylococci is not known, its properties as studied are considered below.

The size of the molecule is such that it does not pass through a collodion membrane. The agglutinating principle is not soluble in chloroform, alcohol, ether, or acetone. It is destroyed rapidly by heating to 46 C and decreased in potency at 40 C. The energy involved in this process is of the order of 120 kilocalories per mole. Pollard and Forro (1949) report that the activation energy for the inactivation of T_1 bacteriophage is 73.6 kilocalories per mole, whereas Kunitz (1948) has shown it to be 57 kilocalories per mole for crystalline soybean trypsin inhibitor protein. Eyring and Stearn (1939) have presented inactivation energies for egg albumin and hemoglobin when these substances are subjected to a variety of catalytic conditions.

Ultraviolet light does not alter the activity of the agglutinating principle under the conditions of our experiments. The active substance was precipitated from concentrated dialyzed chorioallantoic fluid by $\frac{1}{4}$ saturation with ammonium sulfate. Although the biuret test for protein was negative in this fraction, it is conceivable that some protein was there, since this test is not very sensitive. Like the concentrated fluid before separation, the $\frac{1}{4}$ saturation fraction contained no carbohydrate as shown by a negative Molisch test; no urates, as the murexide test was negative and the absorption at 290 mμ was minimal; and

only a suggestion of the presence of phenolic OH groups, since the phenol reagent test yielded only a faintly positive reaction. Crystalline trypsin destroyed the agglutinating principle almost immediately on contact.

It is obvious from our studies in general and from the spectrophotometric examinations in particular that this principle varies in quantity from sample to sample. Further, the material obtained by $\frac{1}{4}$ ammonium sulfate saturation of the concentrated, dialyzed chorioallantoic fluid is not homogeneous and the quantity of associated proteins are not the same from one sample to the next. However, from the configuration of the ultraviolet absorption curve of the $\frac{1}{4}$-saturation fraction, the compound responsible for agglutination is so constituted that when active its absorption at 245 mμ is less than after it has been inactivated. In addition, the absorption in the 260-mμ region, together with the action of trypsin on the principle, suggests that the principle may be protein in nature. From the observations above on the heat of inactivation, one might say that the agglutinating principle in chorioallantoic fluid is a relatively unstable molecule at temperatures over 41 C. It is not soluble in lipid solvents.

How the agglutinating principle acts, and why it is greater in influenza-virus-infected fluid, are questions that cannot be answered at present. Further, there is no explanation as to why only approximately 46 per cent of the strains of *Staphylococcus aureus* are agglutinated by this substance. The solution of all of these problems must await further investigation.

A comparison of the properties of this material with those of lysozyme emphasizes that the agglutinating principle is not related to the latter (Fleming, 1932–1933; Alderton, Ward, and Ferold, 1945). Lysozyme is considerably more stable to heat and acts best at neutrality, its activity being reduced both on the acid and alkaline side.

SUMMARY AND CONCLUSIONS

The properties of the principle in chorioallantoic fluid that agglutinates *Staphylococcus aureus* strains have been studied. This principle is not soluble in the common fat solvents, nor does it contain polysaccharide. It does contain nitrogen, and from its ultraviolet absorption curves, as well as from the fact that it is promptly destroyed by trypsin, one may conclude that the principle is protein in nature. The molecule is labile at temperatures over 41 C and requires an activation energy of 120 kilocalories per mole to inactivate its biological effect.

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INHIBITION OF BACTERIAL ESTERASES BY CHLORAMPHENICOL (CHLOROMYCETIN[1])

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Recently a series of experiments have been undertaken to determine the interrelationships between bacterial enzymes and the new antibiotic chloramphenicol. It was hoped that some inhibitory action of this drug on a given enzymatic system could be detected which might help to explain the mode of action of the compound on bacterial cells. At the present time the inhibitory action of chloramphenicol has been tested on approximately 45 isolated enzymatic systems. The results obtained in our laboratory from these experiments have demonstrated that the antibiotic does not inhibit cellular respiration of either resting or actively growing bacterial cells. The utilization of carbohydrates with the production of organic acids by way of the phosphorylation cycle was also not blocked (Smith and Worrel, unpublished). Furthermore, the breakdown of proteins into amino acids and their transamination were not influenced (Smith and Worrel, in press). These studies and related observations on the inhibitory action of chloramphenicol on phosphatases, nucleases, and oxidizing enzymes suggested the possibility that chloramphenicol might be inhibiting the metabolism of fats and related organic acid esters. Two possible types of reactions would be involved in this general metabolic process, namely, the hydrolysis of the fat or ester and the utilization of the resulting organic acid and alcohol. The present paper deals with a few of the observations made on the inhibitory action of chloramphenicol on bacterial esterases that are involved in the hydrolysis and synthesis of fatty acid esters.

METHODS

The inhibitory actions of chloramphenicol on *Escherichia coli* esterase, crystalline horse liver esterase, and the esterase activity of mitochondria were studied by a method essentially the same as that reported by Singer and Hofstee (1948). The method as routinely used in the course of the present study was as follows: In the main compartment of a conventional Warburg vessel was placed 0.5 ml of 0.2 M tributyrin emulsified with 2 per cent gum acacia in 0.025 N NaHCO$_3$ buffer equilibrated with CO$_2$ to give a pH of 7.40 at 38 C, 2 ml of the 0.025 N NaHCO$_3$ buffer, and 0.5 ml of the chloramphenicol solution. In one side arm was placed 0.5 ml of the enzyme solution and in the other side arm 0.5 ml of concentrated HCl, which was used to determine the quantity of CO$_2$ retained by the solutions. This additional precaution was only necessary when crude enzyme preparations were used and could be omitted when highly purified samples of esterase or lipase were used. The vessels were aerated with 5 per

[1] Parke, Davis and Company's trademark for chloramphenicol.

cent CO_2 and 95 per cent N_2 gas for 5 minutes, followed by a temperature equilibration for 10 minutes. All determinations were run at 38 C. The chloramphenicol solutions ranging in concentrations from 0.01 μg to 3 mg per ml were used in these experiments.

The inhibitory action of the drug was tested on the bacterial esterase activity of the living *E. coli* cells and on preparations of the bacterial esterase prepared according to the procedure of Tammisto (1933). The action of the antibiotic on bacterial esterase was then compared with its action on crystalline horse liver esterase (Mohamed, 1948) and mitochondria (Hogeboom *et al.*, 1948). The suspensions of the bacterial cells used in these studies as the enzyme solutions were prepared by suspending the freshly harvested cells in 0.025 N $NaHCO_3$ buffer, pH 7.4. The cells were grown for 16 to 18 hours at 38 C in a peptone beef broth containing 3 g Difco beef extract, 10 g peptone, 20 g glucose, and 5 g sodium chloride per liter of solution. The cells were harvested by passing the culture through a Sharples centrifuge at 25,000 rpm and washed four times with neutral 0.8 per cent sodium chloride solution and finally with cold 0.025 N $NaHCO_3$ buffer. The cells were then suspended in the buffer and stored at 0 C until used. Freshly prepared suspensions were used in each determination.

The manometric readings were made at 10-minute intervals. The values reported in this paper are the average of the readings taken between the 10- and 20-minute intervals for 2 to 4 sets of duplicate experiments. By the use of readings from this time interval, any discrepancies due to traces of $(NH_4)_2SO_4$ which might remain in the enzyme preparations or slight variations in the pH values of the solutions used, which would cause a slight variation in the initial manometric readings, would be accounted for in the first readings and would not be included in the differences between the first and second readings.

EXPERIMENTAL RESULTS

The actions of chloramphenicol on bacterial esterase were studied at concentration levels ranging from 0 to 400 μg per ml of the final solution. This broad range includes those concentrations of the drug that are both subbacteriostatic and those that are clinically effective. At least four definite responses to various concentrations of chloramphenicol were observed, as are indicated in figure 1. At low concentrations (0.8 to 1.0 μg) of the drug, there was no significant change in the esterase activity of the *E. coli* cells. With slightly higher concentrations (1 to 3 μg) there appeared to be a definite inhibition of enzymatic activity. In some cases decreases as much as 25 per cent in the enzymatic activity could be observed. With still higher concentrations of 3 to 50 μg, there was a marked stimulation of the enzymatic activity. The extent of this stimulation varied from preparation to preparation, but in general there was from 50 to 80 per cent increase in activity. When the concentration of chloramphenicol was increased beyond 50 μg, there was a definite and almost complete inactivation of esterase activity. In most cases only slight traces of esterase activity could be detected. A detailed study of each portion of this general curve showed a constant relationship between enzymatic activity and chloramphenicol concentration.

The various response zones described above can also be easily seen on the culture plates used in the assay of chloramphenicol. These plates, prepared according to the FDA procedure for the assay of streptomycin and spotted with chloramphenicol, show at least three zones of growth. In the center zone growth is completely inhibited and the area appears translucent. This zone of inhibition is surrounded by the ring of enhanced growth, in which the growth of the bacteria has been stimulated by subbacteriostatic concentrations of the drug. This area presents a distinct contrast to the background area, where there is normal growth of the bacteria. If the method of Knaysi (1941) is used to detect the esterase activity in each of these zones, it will be seen that the zone of in-
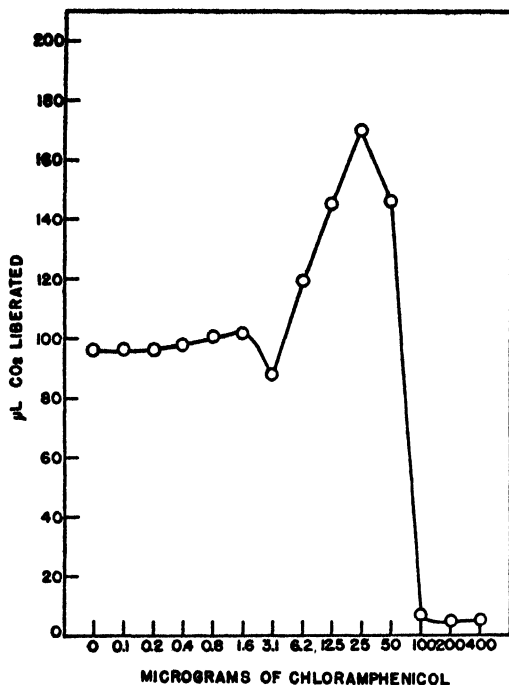
Figure 1. The influence of chloramphenicol on the esterase activity of *E. coli* cells.

hibition is completely lacking in esterase activity, whereas the ring of enhanced growth exhibits maximum enzymatic activity. Furthermore, the esterase activity of the enhanced growth ring is more intense than the activity of the general background area.

In connection with the effects of various concentrations of chloramphenicol on the esterase activity of *E. coli* cells, it is interesting to note the similarities between the esterase inhibition curve (figure 1) and the typical growth curve (figure 2) obtained when this organism is grown under the influence of various concentrations of the antibiotic. In the experiments reported in figure 1, approximately ten times the number of bacteria were employed as the source of the enzyme at each concentration of the drug as were present in the cultures

of the experiments reported in figure 2. Therefore, both curves show the same responses at approximately the same concentrations of chloramphenicol. The curves indicate that low concentrations of the antibiotic produced no measurable change in either growth or esterase activity. With higher concentration there was a slight stimulation of both processes, followed by an inhibition. This was in turn followed by a marked stimulation and finally by inhibition.

The similarities in these results are even more interesting when one recalls that all concentrations used were well within the range of concentrations of the drug which can be maintained in the blood and which are clinically effective.
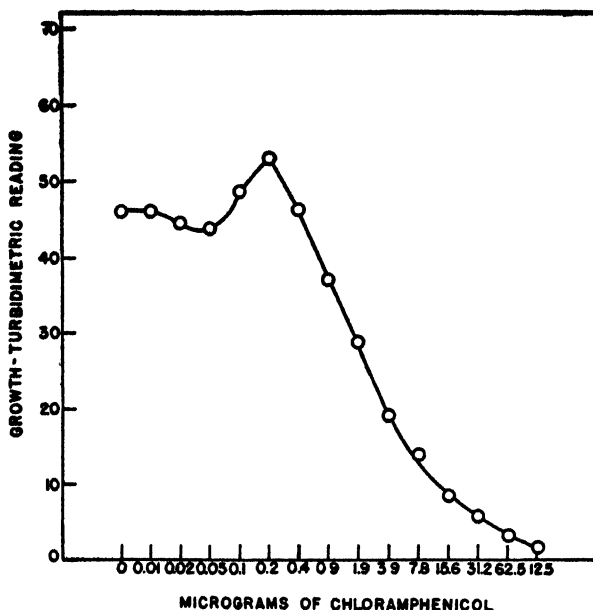
Figure 2. The influence of chloramphenicol on the growth of *E. coli*.

Essentially the same responses were observed from the studies of the effects of chloramphenicol on crystalline horse liver esterase (figure 3) as were observed in the studies of bacterial esterase. There were, however, some differences in the magnitude of the responses exhibited by this esterase and by the bacterial esterase. In general, the inhibition effects at low concentrations were more pronounced and the stimulation effects at higher concentrations were less pronounced with the horse liver esterase than with the bacterial esterase.

The antiesterase action of the drug was also tested on the bacterial esterases prepared from *Bacillus mycoides*, *Bacillus subtilis*, and *Proteus vulgaris*. The responses observed with these esterase preparations were for all purposes identical with the responses described above. Highly purified preparations of lipase (Glick and King, 1933) and crystalline beef liver esterase (Mohamed, 1948) gave similar results.

The antiesterase effects of chloramphenicol on mitochondria (Hogeboom *et al.*, 1948) and liver homogenates (Potter and Elvehjem, 1936) were noticeably different from those reported above. The antiesterase action of the drug as far as bacteria are concerned is excellent, but when mitochondria or animal cells are used the action is incomplete. The drug inhibits only 40 to 50 per cent of the esterase activity exhibited by mitochondria (see figure 4). Furthermore, at high concentrations of the antibiotic, 100 μg or more, no significant inhibition of the esterase could be detected.

The observations reported above are reminiscent of the work of Umbreit (1949) and Umbreit and Tonhazy (1949) on the action of streptomycin. The results suggest that chloramphenicol will inhibit the esterase within the animal
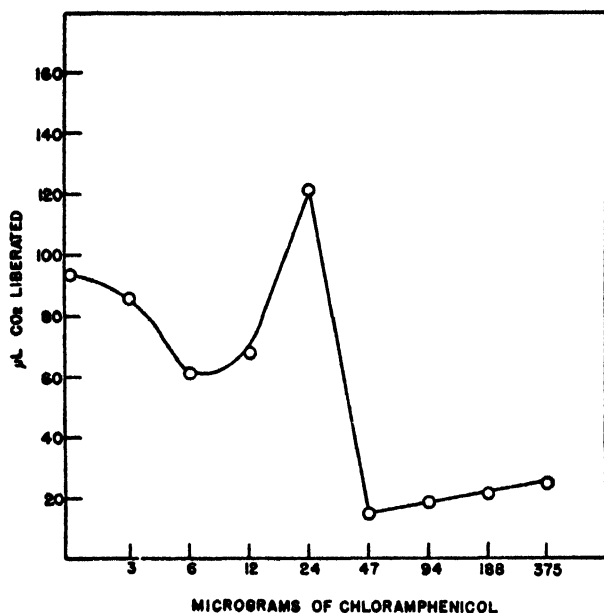
Figure 3. The influence of chloramphenicol on crystalline horse liver esterase.

cell as well as within the bacteria but that in the animal there is some barrier at the cell wall and at the mitochondria which prevents the chloramphenicol from reacting with the esterase within the cell.

The observations that have been presented are the only indications that have been encountered in a study of some 45 enzymatic systems known to occur in *E. coli* in which chloramphenicol exhibits an inhibitory effect against a given enzymatic system at concentrations that would be clinically effective. These results suggest that chloramphenicol might be acting through its effects on the esterase systems present in bacteria. It does not mean, however, that this is the only reaction that might be involved in the bacteriostatic action of the drug. This basic finding might, however, be a clue to the action of chloramphenicol on pathogenic bacteria. In order to provide a satisfactory explanation of the

mode of action of chloramphenicol in therapeutic practice, it will be necessary to demonstrate how the antibiotic can enter the animal body and overcome the pathogenic bacteria without injuring the animal tissues. The first step in this over-all problem has been to demonstrate which reactions of the bacterium are inhibited by concentrations of chloramphenicol that would be clinically effective. The next step will be to determine how the antibiotic inhibits the bacterial esterase and still is prevented from reacting with the tissue esterases that the drug will inhibit if allowed to come in contact with them. Finally the last problem will be to explain the mechanism by which resistant strains of the organism can overcome the inhibitory action of the antibiotic. The last two problems are now under investigation in our laboratory and the results will be reported later.
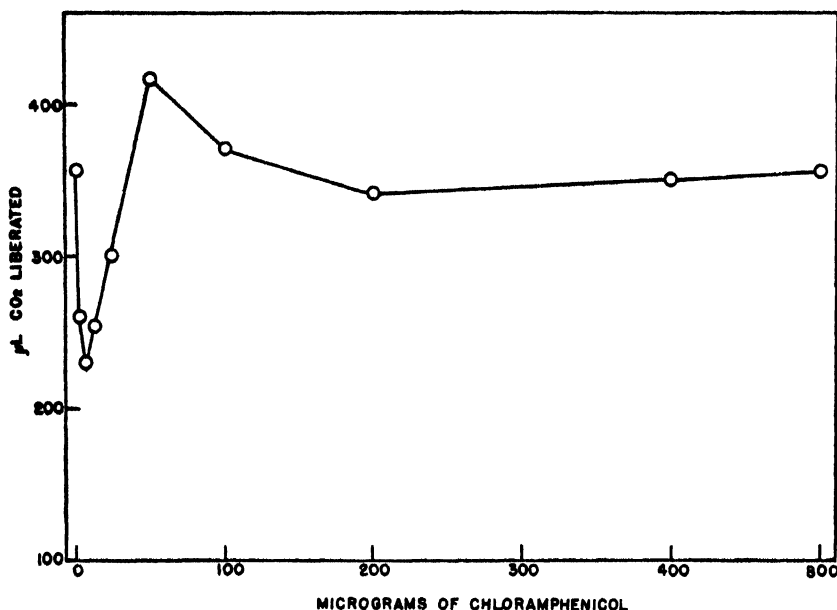
Figure 4. The influence of chloramphenicol on the esterase activity of mitochondria.

SUMMARY

Studies on the mode of action of chloramphenicol at therapeutic levels indicate that the ability of this new antibiotic to inhibit the normal metabolic processes of pathogenic bacteria may be related to its inhibitory action on esterase. Data have been presented to show that the antiesterase action of the drug as far as bacterial and crystalline liver esterase are concerned is excellent, but when mitochondria or animal cells are used the action is incomplete. The observations suggest that some barrier exists at the cell wall and at the mitochondria which prevents the chloramphenicol from reacting with the esterase within the animal cell.

There is remarkable agreement between the effects of various concentrations

of chloramphenicol on the growth and esterase activity of *Escherichia coli* cells. At least four distinct responses have been observed. At extremely low concentrations the drug produces no significant change in either growth or esterase activity. With higher concentrations a slight inhibitory effect is observed. This in turn is followed by a marked activation of both processes. In the fourth concentration range, which is the therapeutic range, there is a marked inhibition of both growth and esterase activity. This inhibitory action of chloramphenicol on esterase activity may therefore be a clue to the bacteriostatic action of this antibiotic.

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COBALT AND BACTERIAL GROWTH, WITH SPECIAL REFERENCE TO PROTEUS VULGARIS[1]

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For the past five years we have been engaged in an investigation of the effect of cobalt on the growth and metabolism of bacteria, especially of *Proteus vulgaris*. The emphasis on *Proteus* followed requests for aid in the control of growth of this penicillin- and sulfa-resistant organism in cases of peritonitis, cystitis, and eye infections.

Interest in cobalt as a growth inhibitor of microorganisms has been extremely limited. Such studies as have been made deal primarily with the concentrations of the metal necessary to kill the cells of bacteria, yeast, or paramecia after a given period of exposure (Bokorny, 1905, 1913; Krauss and Collier, 1931; Johnson, Carver, and Harryman, 1942). Apart from observations on the therapeutic use of cobalt in the treatment of tuberculosis (Renon, 1915; Rondoni, 1920; Mascherpa, 1929), no attempt, so far as we are aware, has been made to study in detail the nature of the action of cobalt on bacteria.

In this paper we shall consider only those aspects of the growth-inhibitory effect of cobalt on bacteria, particularly *P. vulgaris*, which are of a cultural rather than of a metabolic nature. The results of our metabolic studies will appear subsequently.

EXPERIMENTAL RESULTS

Bacterial spectrum. In the course of an investigation of the elements with which conalbumin might combine to account for its growth-inhibitory action on a culture of *Shigella dysenteriae* (Schade and Caroline, 1944), we observed that the test organism failed to grow in nutrient broth to which 10 to 20 ppm cobalt had been added. This observation, as well as the lack of information regarding the growth-inhibiting properties of cobalt, prompted us to make a preliminary survey of the response of a limited number of bacteria to cobalt additions to their culture media.

Table 1 lists the microorganisms tested, their source, and the concentration of cobalt required to inhibit growth completely. Among the bacteria are representative gram-positive and gram-negative, as well as aerobic and anaerobic, species. The media in which the cultures are grown are likewise detailed, since later work has shown that the composition of the medium is important for the consideration of the minimum amount of cobalt needed for effective growth inhibition.

That different strains of a given species may vary with respect to the concentration of cobalt required to effect complete inhibition of growth is readily

TABLE 1

List of microorganisms tested for growth inhibition by cobalt

ORGANISM	SOURCE	COBALT (PPM)*
Proteus vulgaris (2 strains)	Cystitis	20–30
Proteus vulgaris (3 strains)	Sinusitis	30–50
Escherichia coli	Ulcerative colitis	10–20
Escherichia coli (2 strains)	Sinusitis	70–100
Salmonella paratyphi A	Stock culture	20–30
Salmonella typhimurium	Stock culture	30–50
Salmonella cholerae-suis	Stock culture	10–20
Salmonella enteriditis	Stock culture	30–50
Salmonella pullorum	Stock culture	10–20
Salmonella newington	Stock culture	30–5
Shigella dysenteriae (3 strains)	Stock culture	10–20
Shigella dysenteriae (3 strains)	Stock culture	20–30
Shigella paradysenteriae (2 strains)	Stock culture	1–10
Shigella paradysenteriae (1 strain)	Stock culture	10–20
Shigella paradysenteriae (1 strain)	Stock culture	50–100
Shigella sonnei	Stock culture	50–100
Shigella dysenteriae sp. (Newcastle)	Stock culture	10–20
Alcaligenes faecalis (7 strains)	A.T.C.C.	1–10[1]
Staphylococcus aureus (4 strains)	Stock culture	30–50
Staphylococcus aureus (2 strains)	Stock culture	10–20
Staphylococcus albus	Stock culture	10–20
Staphylococcus albus	Stock culture	75–100
Streptococcus faecalis	Stock culture	40–60
Streptococcus viridans	Stock culture	40–60[2]
Clostridium septicum	Stock culture	10–20[3]
Clostridium chauvei	Stock culture	10–20[3]
Clostridium pasteurianum	A.T.C. 7040	20–30[4]
Saccaromyces cerevisiae	Fleischman strain 139	100–125[5]
Leptomitus lacteus (Roth) Agardh		5–10[6]

* Concentration of cobalt required to effect complete inhibition of growth in nutrient broth (0.5 per cent meat extract and 1.0 per cent peptone in 0.5 per cent saline, pH 7.3) except when otherwise noted.

[1] Five-tenth per cent sodium lactate, 0.1 per cent ammonium sulfate, 0.01 per cent magnesium sulfate heptahydrate, in 0.025 M phosphate buffer.

[2] Nutrient broth plus blood serum.

[3] Nutrient broth plus 0.1 per cent sodium thioglycolate and 0.1 per cent brain heart infusion.

[4] One per cent malt extract plus 0.1 per cent yeast extract.

[5] Nutrient broth plus 0.05 per cent yeast extract and 1 per cent glucose.

[6] One-tenth per cent L-leucine, 0.05 per cent DL-alanine, 0.05 per cent glutamic acid, 0.0005 M $MgSO_4$, and 0.005 M phosphate buffer, pH 6.6.

apparent from a study of the seven A.T.C. strains of *Alcaligenes faecalis* investigated. Three strains (4741, 8749, and 8750) were inhibited by 1 ppm cobalt;

one strain (213) by 2 ppm cobalt; two strains (8748 and 9220) by 4 ppm cobalt; and one strain (212) by 7 ppm cobalt. Further, we have been able to train an isolate of a strain of *P. vulgaris* whose growth is halted by 50 ppm cobalt to grow well at 150 ppm and to require at least 200 ppm cobalt for effective inhibition.

Of the various bacteria listed in table 1, we chose to concentrate our attention upon the species *P. vulgaris* as a test organism for an extensive study of the action of cobalt on growth and metabolism. The strain of *Proteus* we employed had been isolated from a cystitis case.[3] When we found that this organism failed to grow in nutrient broth containing 30 ppm cobalt, it was of added interest to find that its growth in urine was likewise inhibited by cobalt.
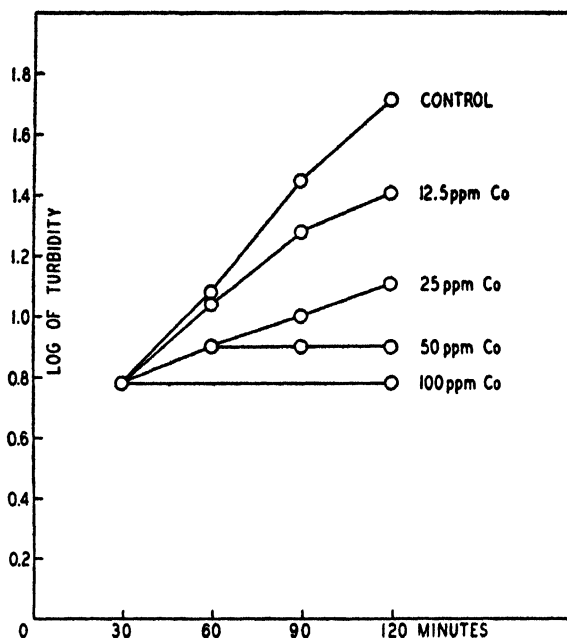
Figure 1. Growth rate of *P. vulgaris* in nutrient broth as affected by the addition of increasing concentrations of cobalt to cultures in early logarithmic phase of growth.

Growth-inhibiting concentrations of cobalt and composition of the growth medium. When increasing concentrations of cobalt were added to young cultures of *P. vulgaris* growing in nutrient broth, the subsequent growth rates of the cultures were increasingly affected. Figure 1 summarizes typical results obtained with such cultures to which 0, 12.5, 25, 50, and 100 ppm cobalt had been added. In addition to the fact that greater concentrations of cobalt effect greater inhibitions of the rate of growth, it is apparent that 50 ppm cobalt, though adequate finally to inhibit growth completely, are not immediately effective as is a concentration of 100 ppm.

[3] Kindly supplied by Dr. G. Schwartzman, Mt. Sinai Hospital, New York.

In a preliminary attempt to determine whether cobalt was exerting its effect on the growth of *Proteus* through interference with the utilization by the organism of some nutrient in the medium essential to it as a growth factor, nicotinamide (Peterson and Peterson, 1945) and magnesium sulfate (Knight, 1936) were added to the nutrient broth. Such additions had no effect on the concentration of cobalt required to bring about cessation of growth. Additions of glucose and of ammonium sulfate to the medium were also ineffective.

That the concentration of cobalt needed to stop the growth of *P. vulgaris* in nutrient broth was, however, related, in a large measure, to the combination of cobalt with some constituent or constituents in the medium became evident when a determination was made of the cobalt needed for growth inhibition of this bacterium cultured in a synthetic medium. Our strain was adapted to grow in the following synthetic medium: 0.05 M glucose, 0.0075 M ammonium sulfate, 0.0004 M magnesium sulfate heptahydrate, and 0.025 M potassium phosphate buffer at pH 7.3. A concentration of cobalt of about 0.25 ppm prevented growth in this medium as judged by turbidity in comparison with about 100 times this concentration in nutrient broth and 1,000 to 2,000 times in brain heart medium (Difco).

When brain heart infusion medium was dialyzed to remove low molecular weight constituents and then added to nutrient broth, cobalt, at the same concentration as was required in the broth alone, inhibited growth of *P. vulgaris*. Addition of the ash of the brain heart infusion medium was likewise without effect on the concentration of cobalt required to stop growth of the bacterium in nutrient broth. These observations indicated that the amino acid component of the brain heart medium and, probably, of the nutrient broth itself might be responsible through some cobalt-amino-acid complex formation for the need of higher concentrations of cobalt to effect inhibition of growth. To test this hypothesis we investigated the effect of the addition of casein hydrolyzate (Smaco vitamin-free casein hydrolyzate) to nutrient broth at a concentration level of 30 mg of hydrolyzate per ml of medium on the inhibition by 50 ppm cobalt of the growth of *P. vulgaris* at pH 7.2. Under these conditions, cobalt did not interfere with growth of the bacteria.

With the effective concentration of casein as a guide, individual amino acids in concentrations equivalent to those found in casein were next added to nutrient broth to observe which amino acid, if any, might be responsible for the "protection" afforded to the bacteria by casein against the inhibitive action of cobalt. The following amino acids and amines were tested against 50 ppm cobalt in nutrient broth at pH 7.0 to 7.5 inoculated with *P. vulgaris* and incubated for 24 hours at 37 C: DL-alanine, L-arginine, L-aspartic acid, L-cysteine, L-cystine, L-glutamic acid, glutamine, glycine, L-histidine, histamine, L-leucine, L-lysine, DL-methionine, DL-phenylalanine, L-proline, DL-serine, L-tryptophan, L-tyrosine, and DL-valine. In every case the molar ratio of amino acid to cobalt was no less than 4 to 1. Of these 17 amino acids, only L-cysteine and L-histidine prevented or reversed the growth inhibition by cobalt. An indication that the pH of the medium was an important factor in the tests as run became evident when histamine and L-glutamic acid were added to media at pH 8.0 and above. Under

such alkaline conditions these compounds were likewise effective against the inhibitory action of cobalt.

Since cobalt has been shown to form a stable complex with cysteine (Michaelis and Barron, 1929), its effectiveness as a "protective" agent might well have been anticipated. The results obtained with L-histidine, on the other hand, were not expected. Since the casein hydrolyzate employed in these studies did not contain cysteine, it is probable that the "protective" action of the hydrolyzate against cobalt was greatly, if not completely, due to its histidine content.

Reversal of cobalt inhibition by histidine. The studies already published on the characteristics of the cobalt-histidine complex as well as some of the physiological investigations that have made use of the cobalt-combining properties of histidine

TABLE 2

Titration of L-histidine HCl for ability to overcome inhibition of 5 μg cobalt per ml in a synthetic medium

CONC. COBALT	CONC. HISTIDINE HCl	MOLECULAR RATIO HISTIDINE:COBALT	GROWTH
μg/ml	μg/ml		
0	0	—	4+:4+
5	0	—	— —
5	5	0.28	0:0
5	10	0.57	0:0
5	15	0.84	0:0
5	20	1.12	0:0
5	25	1.40	0:0
5	30	1.68	0:0
5	35	1.96	4+:4+
5	40	2.25	4+:4+

Four ml of synthetic media (0.05 M glucose, 0.0075 M ammonium sulfate, 0.004 M magnesium sulfate, and 0.025 M potassium phosphate buffer, pH 7.3) containing 6.25 μg cobalt per ml, as cobalt sulfate, and varying amounts of L-histidine hydrochloride monohydrate were inoculated in duplicate with 0.1-ml suspensions of *P. vulgaris* grown in synthetic medium for 48 hours at 37 C and brought to a final volume of 5 ml with distilled water. Duplicate controls were run in synthetic medium minus cobalt. Readings were taken after 96 hours of culture at 37 C.

(Burk *et al.*, 1946; Hearon *et al.*, 1947) had their genesis in the currently reported bacteriological work and have summarized some of its findings. It is useful, however, to detail an example of the type of experiment which showed the "protective" effect of histidine against the growth-inhibitory action of cobalt on *P. vulgaris* and indicated quite clearly the combining ratio of histidine to cobalt as 2:1 under the particular conditions given. Table 2 illustrates such an experiment. Although in this case a synthetic medium was chosen for the test, similar results were obtained in straight nutrient broth or in nutrient broth diluted 1 to 5. Changing the sequence of addition of cobalt and histidine had no effect on the results.

Evidence that histidine is capable of reversing an established growth inhibition by cobalt is presented in figure 2. Bacteria from an 18-hour culture of *P.*

vulgaris were inoculated into nutrient broth and dispensed in appropriate amounts into Warburg respirometer vessels. Using the rate of respiration as an index of the course of growth, we added cobalt (final concentration = 60 ppm) to all of the cultures other than the histidine-containing controls when the bacteria were leaving their lag phase and entering logarithmic growth. At 1-hour intervals, an amount of histidine sufficient to counteract the concentration of cobalt employed was added to the inhibited cultures. One set of cultures was maintained as a cobalt-inhibited control.

Figure 2 shows that, in each of the four sets of cultures to which histidine had been added, growth of *P. vulgaris* subsequently ensued. The cultures that were inhibited for 1 hour by cobalt required approximately 80 minutes following
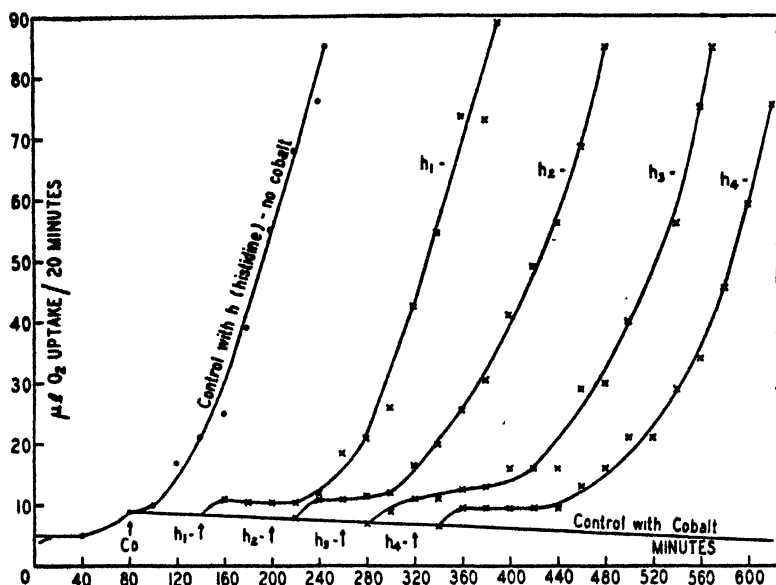
Figure 2. Reversal by histidine of the bacteriostatic effect of 1×10^{-3} M cobalt on *P. vulgaris* in nutrient broth. The histidine was added one (h_1), two (h_2), three (h_3), and four (h_4) hours after inhibition of growth by the cobalt had been effected.

histidine addition to re-emerge from the lag phase. Those that were inhibited for 4 hours required approximately 140 minutes to achieve the same stage of development.

Reactions of cobalt and histidine. The addition of cobalt to a solution of histidine under aerobic conditions resulted in the formation of a progressively deepening yellow-brown color that could be dissipated by acidification and reformed through subsequent neutralization. When the colored mixture was kept overnight at room temperature, its yellow-brown hue had changed to reddish. Under anaerobic conditions the yellow-brown or reddish color failed to develop in the mixture of cobalt and histidine, which remained very light pink until oxygen was admitted to the reaction flask. Similar color changes occurred

upon the addition of cobalt to nutrient broth in the absence of added histidine. It was observed, further, that when cobalt was added to either nutrient broth or a solution of histidine, a fall in the pH of the mixture took place.

The chemical studies that have been made of the cobalt-histidine complex formation (Hearon, 1948; Hearon, Burk, and Schade, 1949) have elucidated the significance of these observations. In summary, two molecules of histidine combine immediately and reversibly with one atom of cobalt to yield one molecule of cobaltodihistidine and two hydrogen ions. The formation of the cobaltodihistidine complex is responsible for the pink color of the cobalt-histidine mixture under anaerobic conditions and for its acid pH shift. In the presence of oxygen, two molecules of cobaltodihistidine combine rapidly and reversibly with one molecule of oxygen to form one molecule of oxy-*bis*(cobaltodihistidine), which complex imparts the yellow-brown color to the solution. Slowly, with or without the intervention of additional oxygen, the oxy-*bis*(cobaltodihistidine) changes to an irreversibly oxygenated form with no evident oxidation of cobaltous to cobaltic. This irreversible form is reddish pink in color.

Cobalt inhibition under aerobic and anaerobic conditions. Although it is clear from table 1 that aerobic and anaerobic bacterial species are sensitive to cobalt concentrations of the same order of magnitude, it is not evident that a given species grown under both aerobic and anaerobic conditions would respond to cobalt in a comparable manner. To investigate this point, studies were made of the sensitivity to cobalt of *P. vulgaris* and *Staphylococcus aureus* grown under aerobic and anaerobic conditions in nutrient broth. With both bacterial species, irrespective of the presence or absence of oxygen, the rates of growth are initially affected by the same cobalt concentrations. To achieve complete inhibition, however, roughly two to three times as much cobalt is required under anaerobic as under aerobic conditions. Although it is possible that different metabolic systems are affected by cobalt depending upon the presence or absence of oxygen, we believe that cobalt is exerting its inhibitive effect on a common metabolic system in the cells of these two bacterial species independently of oxygen.

Effect of pH of medium and temperature of incubation on cobalt inhibition. To determine the effect of pH of the medium on the growth-inhibitory action of cobalt on *P. vulgaris,* we set up cultures of this organism in nutrient broth diluted 1:5 with distilled water, and adjusted with HCl to pH's 7.3, 6.1, and 5.6. To each medium enough cobalt was added to give a final concentration of 10 ppm, which concentration had previously been shown to be just adequate to effect complete inhibition over a pH range of 7.0 to 7.5. Inocula were then added to the pH-adjusted, cobalt-containing media and incubated at 37 C over a period of 96 hours. The results of two experiments in which the tests were run in duplicate failed to show any effect of the pH levels employed on the inhibitory action of cobalt.

The effect of the temperature of incubation of *P. vulgaris* cultures on cobalt inhibition of growth was investigated by setting up series of test tubes containing synthetic medium and·concentrations of cobalt ranging from 0.2 to 5 ppm. After inoculation, duplicate series of tubes were put at 37 C and 20 C to

incubate. After making due allowance for the greater length of time required for the bacterial cells at 20 C to grow to the same extent as those incubated at 37 C, we found that the same concentration of cobalt was required to effect complete inhibition of growth regardless of the temperature of incubation.

Effect of cell number on cobalt inhibition. In the investigation of the several factors that affect the minimum concentration of cobalt required to bring about complete inhibition of the growth of *P. vulgaris*, the inocula employed were standardized roughly with respect to the number of bacteria per ml of the test growth medium. Qualitative evidence had, indeed, suggested that the values of the limiting concentrations of cobalt varied with the initial number of cells present in the test medium. To obtain quantitative data on this point, we set up experiments in which the initial number of cells varied from 2×10^4 to 2×10^7 per ml and from 2×10^2 to 2×10^4 per ml of synthetic medium at cobalt levels of 1.2 ppm and 0.12 ppm, respectively. The results showed that the effectiveness of any given cobalt concentration is a function of the number of cells initially present in the inoculum.

Effect of cobalt on size and stainability of cells. We have observed microscopically the effect of cobalt upon the size and stainability of resting cells of *P. vulgaris* following their inoculation into nutrient broth. When cells, grown for 24 hours on a nutrient agar slant at 37 C, were inoculated into nutrient broth and examined over a 2-hour period of incubation at 37 C, they showed an increase in size approximately twice their initial volume in the first hour, usually without evident cell division. During the second hour, division of the cells occurred along with maintenance of their enlarged size. Under otherwise comparable conditions, cells inoculated into nutrient broth containing a concentration of cobalt sufficient to inhibit growth failed to show any increase in size or evidence of cell division over the 2-hour period of incubation. Cells from both the control and cobalt-treated series, on the other hand, when stained with crystal violet, showed similar increases in stainability by the end of the 2-hour incubation period. These results are in conformity with the finding (Levy, Skutch, and Schade, 1949) that the ribonucleic acid concentration of the control and cobalt-inhibited cells increases to approximately the same extent over the same period of time.

Effect of cobalt on viability of cells. Since, as we have shown, histidine is capable of reversing the inhibition of growth of a culture of *P. vulgaris* by cobalt, the question arises to what extent, if any, does cobalt result in the death of the individual members of the population of the bacterial inoculum. Further, is the cell viability dependent upon the stage of the growth cycle from which the inoculum is prepared?

. Studies were made of the effect of cobalt on the number of viable cells remaining in nutrient broth to which a concentration of cobalt had been added sufficient to bring about complete inhibition of growth of the culture. Colony plate counts were made in quadruplicate at two dilutions of the culture at zero time, 30, 120, and 240 minutes following cobalt addition to the broth maintained at 37 C throughout the experiment. In one case, cobalt was added to broth simul-

taneously with the addition of the inoculum of resting cells prepared from an 18-hour nutrient agar slant culture. In the second case, cobalt was added following 30 minutes' incubation of the broth culture at 37 C, at which time the cells were still in the lag phase. In the third case, cobalt was added following 120 minutes' incubation of the broth culture at 37 C, by which time the cells were in their logarithmic growth phase. The results of these experiments are summarized in figure 3. It is clear that the cells of *P. vulgaris* varied in the degree of their sensitivity to a growth-inhibiting concentration of cobalt depending upon the stage of their life cycle at the time of cobalt addition to the growth medium. The viability of cells in the resting stage was relatively little affected by cobalt after a 4-hour exposure to the inhibitor compared to that of cells in their logarithmic growth phase. Cells in their lag phase were of intermediate sensitivity. The correlation of viability sensitivity to cobalt with the stage of the growth cycle
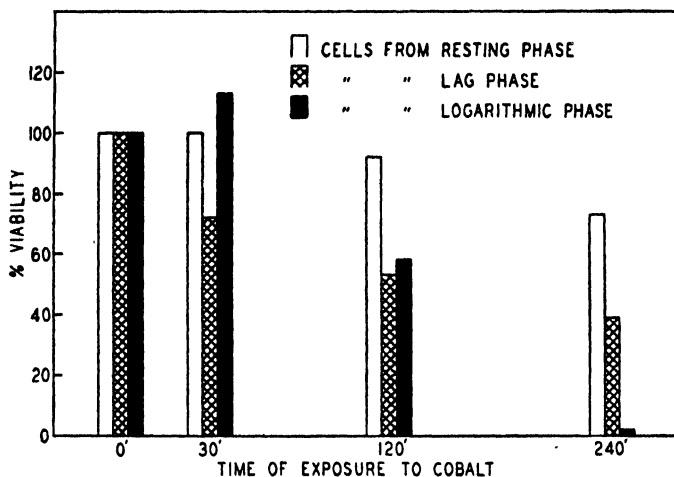
Figure 3. The relation of the viability of cells of *P. vulgaris* in their several growth phases to the length of time of exposure to cobalt in nutrient broth.

is interestingly paralleled by the respiration sensitivity to cobalt of *P. vulgaris* (Schade and Levy, 1949). The respiration of resting cells added to nutrient broth containing cobalt was comparable to that of resting cells added to nutrient broth minus cobalt. The respiration of cells growing logarithmically in nutrient broth, on the other hand, was greatly reduced on the addition of cobalt. Figure 3 also shows that, when cobalt was added to a logarithmically growing culture, not all of the cells at least were immediately inhibited from dividing since the number of viable cells observed one-half hour after cobalt addition to the cultures was greater than at zero time.

It was possible that the decrease in the number of viable cells in nutrient media containing cobalt might be a reflection of a normal rate of death of cells unable, for one reason or another, to grow. To check this possibility, resting cells from an 18-hour culture grown on nutrient agar at 37 C were inoculated into

phosphate buffer and into nutrient broth plus cobalt. Colony plate counts of viable cells from each medium were made over a period of 50 hours' incubation at 37 C. The results are given in table 3 (a). Comparison of the percentage drops in viable cell count shows that cobalt significantly effected a reduction in viability over and above that observed in a medium deficient in nutrients.

Further evidence that cobalt affects viability in a manner other than just to permit continued maintenance of the cell while inhibiting its growth was obtained by suspending resting cells of *P. vulgaris* in phosphate buffer with and without added cobalt. Table 3 (b) summarizes the data. It is again apparent

TABLE 3

Cobalt and the viability of P. vulgaris

(a)

	INCUBATION (HOURS)			
	0	6	26	50
Per cent viability in				
(a) Broth + 60 ppm cobalt	100	15	0.4	0.05
(b) 0.05 M phosphate buffer	100	95	28	22

(b)

	INCUBATION (HOURS)		
	0	5	26
Per cent viability in			
(a) 0.001 M phosphate buffer	100	86.4	19.1
(b) 0.001 M phosphate buffer + 10 ppm cobalt	100	43.2	0.001

Suspensions of a culture of *P. vulgaris*, grown on nutrient agar slant for 18 hours at 37 C, were inoculated into the given media (pH 7.3) to give initial cell concentrations of 2.3 × 10⁵ and 2.2 × 10⁶ for experiments summarized in (a) and (b), respectively. The inoculated media were incubated at 37 C and tested for viable cell number at the stated intervals, by use of the colony plate count method.

that cobalt does, in time, have a significant effect on the viability of these bacteria.

SUMMARY

The growth of representative species of bacteria, both aerobic and anaerobic as well as gram-positive and gram-negative, is completely inhibitable by concentrations of cobalt ranging from 1 to 100 ppm. The actual inhibiting cobalt concentration depends upon the sensitivity of the individual strain of bacterium, the number of cells per ml used as inoculum, and the constituents of the growth medium. As an example of the importance of the last-named factor, the concentration of cobalt necessary to inhibit the growth of *Proteus vulgaris* in meat extract peptone broth is 100 times that required in a synthetic medium of glucose and ammonium sulfate.

Of 17 amino acids tested under physiological conditions of pH and temperature, only histidine and cysteine are capable of overcoming the growth inhibition of *P. vulgaris* by cobalt. For complete prevention or reversal of the growth inhibition, the molar ratio of histidine to cobalt must be at least 2 to 1.

The pH of the medium and the temperature of incubation have no effect on the concentration of cobalt required to inhibit the growth of *P. vulgaris*. Irrespective of the presence or absence of oxygen, the rates of growth of this species and of *Staphylococcus aureus* are initially affected by the same cobalt concentrations. For complete inhibition, approximately two to three times as much cobalt is required under anaerobic as under aerobic conditions.

Cells of *P. vulgaris*, incubated at 37 C for 2 hours in nutrient broth containing an inhibiting concentration of cobalt, fail to show any increase in cell size or evidence of cell division. By the end of the 2-hour incubation period, however, they do show an increase in stainability with crystal violet comparable to that of the controls. If cobalt is added to nutrient media inoculated with cells in their resting, lag, and logarithmic phase of growth, their viability after a period of 4 hours is decreased to 75, 40, and 2 per cent of the initial value, respectively. The rate of reduction in the viability of resting cells effected by cobalt in a nutrient medium or in phosphate buffer is greater than the rate of death of cells in phosphate buffer alone.

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COMPARISON OF LUDLAM'S MEDIUM WITH STAPHYLOCOCCUS MEDIUM NUMBER 110 FOR THE ISOLATION OF STAPHYLOCOCCI THAT CLOT BLOOD

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Ludlam (Monthly Bull. Ministry Health, **8,** 15, 1949) described a medium on which, with few exceptions, only *Staphylococcus aureus* strains that clot blood are able to grow, producing large black colonies. The following modification of that medium was used to compare the number of organisms grown on it with the number of similar organisms grown on staphylococcus medium number 110 (Chapman: Trans. N. Y. Acad. Sci., **9,** 52, 1946). Medium consisting of agar, 25 g; mannitol, 10 g; K_2HPO_4, 5 g; LiCl, 5 g; yeast extract, 3 g; tryptone, 10 g; and water, 1,000 ml was adjusted to pH 9.2, sterilized by boiling, and cooled to 55 C. One ml of 5 per cent potassium tellurite was then added, and the cultures were incubated for 48 hours at 37 C.

Of 232 cultures plated on both media, there was complete agreement in 83 per cent; 5 per cent showed from 1 to 10 colonies on Ludlam's medium but none on no. 110; 6 per cent that did not clot blood showed growths on Ludlam's medium; and one colony on no. 110 belonged to the genus *Flavobacterium*.

Therefore, for the highest recovery of blood-coagulating staphylococci, cultures should be plated on both media. But if only one is to be used, staphylococcus medium no. 110 is preferable because of the several cultures containing a large number of organisms that failed to grow on Ludlam's medium. Also this medium, unlike no. 110, cannot be used for the direct application of confirmatory tests. Most of the instances in which Ludlam's medium alone showed from 1 to 10 colonies are of questionable significance.

BACTERIAL UTILIZATION OF ANIONIC SURFACE-ACTIVE AGENTS

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A recent review by Glassman (Bact. Revs., **12,** 105, 1948) covers the extensive literature that deals with the applications in bacteriology of surface-active agents. These compounds have come into widespread use in recent years, especially as detergents and, in certain instances, as antibacterial agents. Migaki

and McCulloch (J. Bact., **58**, 161, 1949) have shown typical survivor curves for bacteria exposed to surface-active agents and state that the same survivor curve trend was observed in all trials with all types of agents tested and with each culture used.

It has been established that gram-positive bacteria are more sensitive to surface-active agents than are gram-negative forms and that the cationic types are more effective antibacterial agents than are the anionic types. Thus certain anionic agents have been incorporated in media to inhibit selectively gram-positive organisms. We are not familiar, however, with any report of the utilization as a source of material for growth of a surface-active agent by bacteria.

Chance observation of what appeared to be bacterial growth in a bottle of a commercial detergent shampoo led to culturing on solid medium and the recovery of a gram-negative rod with a single polar flagellum. Identification studies suggested that the organism is a member of the genus *Pseudomonas*, although it does not agree fully with any of the species of this genus described in *Bergey's Manual*. Of a number of common sugars tested, the organism fermented only glucose, and that feebly. It grew on solid media in which both carbon and nitrogen were supplied by sodium asparaginate or ammonium lactate, and also with ammonium phosphate as the nitrogen source and either glucose or citrate as the carbon source. No pigment is produced.

Good growth occurred on solid media prepared from distilled water, agar, and about 3 per cent shampoo, both with and without added ammonium phosphate. Inferior growth was obtained on 5 per cent sodium lauryl sulfate agar, either with or without added ammonium phosphate. Growth without added N may be explained by the utilization of atmospheric ammonia, or the presence of traces of the ammonia ion in the completed medium. Growth in liquid media has not been so luxuriant as on solid media.

Single strains from the stock cultures of a number of gram-negative organisms have been tested for growth on shampoo agar and on 1 and 5 per cent sodium lauryl sulfate agar. *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* grew, on each medium. *Proteus vulgaris*, *Aerobacter aerogenes*, *Serratia marcescens*, *Escherichia coli*, and *Proteus morganii* grew on the shampoo medium but not on the sodium lauryl sulfate medium. There was no growth on either medium by *Escherichia freundii*, *Alcaligenes faecalis*, *Achromobacter thalassius*, or *Achromobacter aquamarinus*.

The exact chemical composition of the shampoo is not known. The patent disclosure (U. S. Reissue no. 20,636) reveals that the active principle is an anionic agent of the general type ROX $\left[O\!-\!\overset{\displaystyle O}{\underset{\displaystyle O}{\overset{|}{\underset{|}{S}}}}\!-\!O \right]$ Y, in which R represents an acyl radical of at least 4 carbon atoms, X represents a polyhydroxy substance, and Y is the cation.

The results show that at least two anionic surface-active agents will support growth of some types of bacteria.

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